Genotyping of *Trichomonas vaginalis* in antenatal women from eThekwini

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I Rennisha Chetty declare that:

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Supervisor: Dr Nathlee Abbai

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ABSTRACT

Trichomonas vaginalis is the causative agent of trichomoniasis. The genetic characterisation of *T. vaginalis* isolates reveals significant genetic diversity in this organism. Data on the prevalence of different genotypes of *T. vaginalis* in South African populations is lacking. This study investigated the diversity of *T. vaginalis* in a pregnant population in South Africa. In this study, 362 pregnant women from the King Edward VIII hospital in Durban, South Africa provided vaginal swabs to be tested for the presence of *T. vaginalis*. *T. vaginalis* was detected using the TaqMan assay using commercially available primers and probes specific for this protozoa (Pr04646256_s1). The *actin* gene from *T. vaginalis* was amplified with gene specific primers. The *actin* amplicons were digested with *HindII*, *MseI* and *RsaI* and the banding patterns were compared across the three digests for assignment of genotypes. Phylogenetic analysis was conducted using MEGA. The prevalence of *T. vaginalis* in the study population was 12.9% (47/362). Genotype G was the most frequent genotype in our study population. Genotypes H and I were detected in one sample each. According to the multiple sequence alignments and phylogenetic analysis, a level of diversity was observed across and within genotypes. Four different single nucleotide changes in the *actin* gene were detected. Sample TV358 (genotype H) contained a single amino acid substitution from Glutamine to Lysine. Sample TV184 (genotype G) contained a single amino acid substitution from Glutamic acid to Arginine. Sample TV357 (genotype G) contained two amino acid substitutions, Arginine to Leucine and Glycine to Aspartic acid. Three different genotypes were observed in the pregnant population. Diversity was observed across and within genotypes. The observed diversity can be challenging for future vaccine design and development of antigen-based rapid diagnostic tests for trichomoniasis.

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SYMBOLS AND ABBREVIATIONS

%	percentage
>	greater-than
√	recommended by the guidelines
-	not recommended by the guidelines
°C	degree Celsius
µl	microlitre
BASHH	British Association for Sexual Health and HIV
Bp	base pair
BLAST	Basic Local Alignment Search Tool
BREC	Biomedical Research Ethics Committee
BSA	bovine serum albumin
BV	bacterial vaginosis
$C_3H_3N_3O_2$	5-nitroimidazole
$C_8H_{13}N_3O_4S$	tinidazole
$C_{21}H_{20}BrN_3$	ethidium bromide
CDC	Centres for Disease Control and Prevention
CD4 (cluster of differentiation 4)	cluster of differentiation 4
Ct	Cycle threshold
Cq	quantification cycle
D	day
D	aspartic acid
DNA	Deoxyribonucleic acid
E	glutamic acid
G	gram
G	glycine
H	hour
HIV	Human Immunodeficiency Virus
IGS	intergenic spacer
K	lysine
L	leucine
MCA	Modified Columbian Agar
Mg	milligram
MIC	Minimum Inhibitory Concentration
Min	minute
ML	Maximum likelihood
MLST	Multilocus Sequence Typing
Mz	metronidazole
N	number
NAATs	Nucleic Acid Amplification Tests
NC	Negative control
NCBI	National Centre for Biotechnology Information
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
PFOR	Pyruvate-ferredoxin oxidoreductase
PID	Pelvic inflammatory disease
Q	glutamine
R	arginine
RAPD	Random Amplification of Polymorphic DNA
S	seconds
SCM	School of Clinical Medicine
STI	Sexually Transmitted Infections
TVV	*T. vaginalis* virus
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1
INTRODUCTION

Trichomonas vaginalis is referred to as an anaerobic, flagellated protozoan and a causative agent of trichomoniasis. This particular parasite can be transmitted via sexual intercourse from host to host (Conrad *et al*., 2012). Trichomoniasis is considered to be the most prevalent non-viral sexually transmitted infection (STI) worldwide affecting individuals of all ages, ethnicity and socioeconomic groups (Napierala *et al*., 2010; Conrad *et al*., 2012; Apalata *et al*., 2014, Naidoo, 2015). The World Health Organization (WHO) estimated that in the year 2016 approximately 156 million new cases of trichomoniasis was reported in adults aged between 15-49 years (Rowley *et al*., 2019). *T. vaginalis* can infect both males and females, however the infection rate is much higher in women (Khalili *et al*., 2017).

Trichomoniasis has been reported to have over 276.4 million reported infections worldwide (Ramjee *et al*., 2015). Out of the 276 million cases, 25 million cases have been reported in pregnant women (Salakos *et al*., 2018). Pregnant women infected with *T. vaginalis* have a higher risk of preterm delivery, since *T. vaginalis* causes the premature rupture of membranes. A previous study has shown that pregnant women infected with *T. vaginalis* have a 30% risk of preterm delivery or delivering infants who have a low-birth weight (Cotch *et al*., 1997). *T. vaginalis* infection also contributes to the increase in mother-to-child transmission of HIV (Kock *et al*., 2011).

Many molecular based techniques have been used in the past in order to distinguish *T. vaginalis* strains which include Microsatellite genotyping, Multilocus sequence typing (MLST), polymerase chain reaction (PCR)-hybridization, random amplification of polymorphic DNA (RAPD), PCR-size polymorphism as well as PCR-restriction fragment length polymorphism (PCR-RFLP) (Khalili *et al*., 2017). Actin is one of the structural proteins present in *T. vaginalis*. This protein is well-conserved and ubiquitous in nature, thereby making it a likely option for intra-species molecular identification (Meade and Carlton, 2013). To validate the use of this protein for the genetic characterisation of *T. vaginalis*, Crucitti *et al*., 2008, conducted a study using PCR-RFLP, to identify different genotypes of *T. vaginalis* based on the *actin* gene. The study identified eight *T. vaginalis actin* genotypes among 151 isolates obtained from the Democratic Republic of Congo and Zambia (Crucitti *et al*., 2008). Other studies conducted in Kenya and Iran have successfully identified distinct genotypes of *T. vaginalis* based on digestion of the *actin* gene (Matini *et al*., 2017; Masha *et al*., 2017; Khalili *et al*., 2017; Oliaee *et al*., 2017).

Currently, there is a lack of data on the circulating genotypes of *T. vaginalis* in South African populations, particularly pregnant women. In this study, PCR-RFLP of the *actin* gene was performed in order to identify the different genotypes circulating in our population. Identification of the genotypes will provide a snapshot into the molecular epidemiology of *T. vaginalis* in our region which can be used a foundation for larger epidemiological studies on this pathogen.

CHAPTER 2
LITERATURE REVIEW

1.1 Epidemiology of *Trichomonas vaginalis*

Trichomonas vaginalis is referred to as an anaerobic, flagellated protozoan and the causative agent of trichomoniasis. *T. vaginalis* is known to be a haploid eukaryotic parasite, highly motile and aero-tolerant which simply means that it is unable to use oxygen as a source of growth but can tolerate its presence. This particular parasite can be transmitted via sexual intercourse from host to host (Conrad *et al*., 2012). *T. vaginalis* is known to form a linear, double-stranded RNA virus referred to as the *T. vaginalis* virus (TVV) which may be associated with its virulence (Goodman *et al*., 2011; Conrad *et al*., 2012).

Trichomoniasis is considered to be the most prevalent non-viral sexually transmitted infection (STI) worldwide affecting individuals of all ages, ethnicity and socioeconomic groups (Napierala *et al*., 2010; Conrad *et al*., 2012; Apalata *et al*., 2014, Naidoo, 2015). In 2012, the World Health Organization (WHO) reported a total number of 142.7 million cases for trichomoniasis infection (Figure 1). According to Figure 1, the highest number of cases of trichomoniasis was reported for the Western Pacific region (45.3 million cases), followed by African region (37.4 million cases), American region (27.4 million cases), Eastern Mediterranean region (15.6 million cases), South-East Asia region (13.2 million cases) and lastly the European region (3.8 million cases) (Unemo *et al*., 2017).

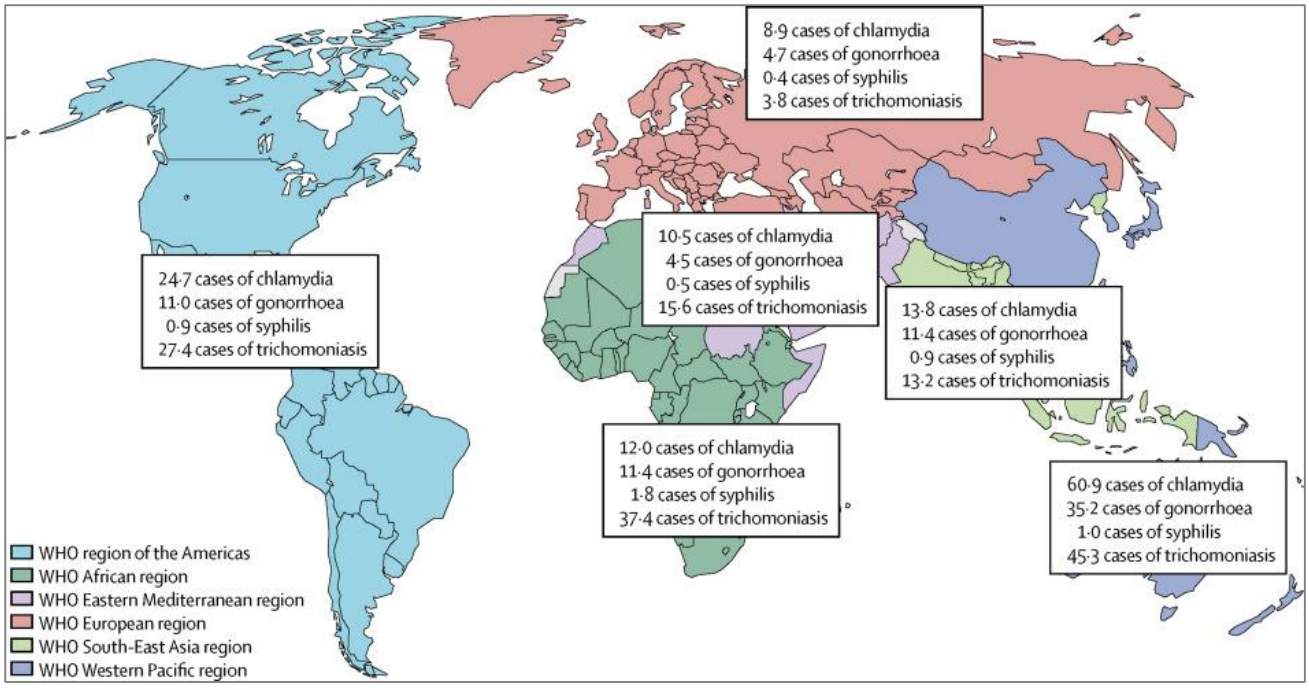

Figure 1: WHO regional estimates of new cases per million of the four curable sexually transmitted infections. Within the African region, the highest number of infections are reported for *T. vaginalis* (Unemo *et al*., 2017).

A study conducted by Newman *et al*., 2015, assessed the global prevalence for trichomoniasis amongst women to be approximately 5.0% (4.0-6.4%) whereas for men it was estimated to be approximately 0.6% (0.4-0.8%) (Newman *et al*., 2015). The lower prevalence of the infection in males is due to the presence of zinc which is found in the prostatic fluid. Zinc is known to have anti-trichomonal activity *in vitro* eventually resulting in the inhibition of *T. vaginalis in vivo* (Krieger and Rein, 1982).

Several studies have reported prevalence data on *T. vaginalis* from South African populations. These studies are described below. Data obtained from a study conducted by Moodley *et al*., 2003, on women attending a reproductive health clinic in KwaZulu-Natal reported a prevalence of 29% for *T. vaginalis*. Another study by Lewis *et al*., 2013 involving both men and women from a primary health care clinic in Johannesburg, found that 23.6% of the women and 6.1%

of the men were infected with *T. vaginalis*. In another study conducted on female sex workers from Durban, the prevalence rate for *T. vaginalis* was 20.3 % (Mlisana *et al*., 2012). Two recent studies conducted by our research group reported prevalence rates of 10% and 13%, respectively for *T. vaginalis* in antenatal women from Durban (Dessai *et al*., 2020, Mabaso *et al*., 2020).

Several studies have identified risk factors for prevalent *T. vaginalis* infection in women. Risk factors include having sex without a condom, ethnicity (more prevalent within the black race group), multiple sexual partners, greater years of sexual activity, history of STIs and previous infection with *T. vaginalis* (Helms *et al*., 2008; Krashin *et al*., 2010). Studies conducted by Abbai *et al*., 2013 and Naidoo *et al*., 2014 in women from KwaZulu-Natal, South Africa found that women 25 years and younger, were at a higher risk of acquiring STIs. Reasons related to younger women being more at risk for infection included behavioural and biological factors (Abbai *et al*., 2013). Abbai *et al*., 2013, also found that women who were previously diagnosed with an STI have a much greater risk for future infection. Other risk factors for acquiring infection included having a lower level of education (Cudmore and Garber, 2010; Abbai *et al*., 2013). A study conducted by Eshete *et al*., 2013 in pregnant women from Ethiopia showed that dysuria, dyspareunia, abnormal vaginal discharge as well as educational status did play a critical role in respect to the increased number of *T.vaginalis* infections. Mabaso *et al*., 2020 also identified abnormal vaginal discharge as a risk factor for *T.vaginalis* infections in pregnant women.

Numerous studies have also shown a positive association between *T. vaginalis* infection and human immunodeficiency virus (HIV) acquisition. *T. vaginalis* increases the risk of acquiring HIV by an estimated 2 fold (Davis *et al*., 2016). This particular parasite has also been associated with an increase in HIV transmission from mother to child (Garber, 2005). The biological reasons for *T. vaginalis* increasing HIV transmission amongst women could be due to two

reasons, (1) the accumulation of both macrophages and CD4 (cluster of differentiation 4) lymphocytes which are HIV target cells and (2) disruption of the vaginal epithelial barrier enabling the movement of HIV into the laminae propriae (Garber, 2005, Abbai *et al*., 2016). Due to the high prevalence of *T. vaginalis* this ultimately leads to a significantly higher number of new HIV infections globally (Conrad *et al*., 2012).

Additionally, in women with trichomoniasis, there is a risk of co-infection with bacterial vaginosis (BV) since *T. vaginalis* has the capability of creating an anaerobic environment resulting in an increase in vaginal pH, thus changing the vaginal flora (Cudmore *et al*., 2004). Gatski *et al*., 2011, reported a co-infection rate of 17.5% for *T. vaginalis* and BV. A later study conducted by Sobel *et al*., 2013, reported a co-infection rate of approximately 70% for *T. vaginalis* and BV. A study conducted by Abbai *et al*., 2016A, in South African women showed a significant association between baseline BV infections and incident *T. vaginalis* infections (Hazard Ratio: 1.60, 95% Confidence Interval: 1.00, 2.57, p=0.04).

1.1.1 History

The observation and description of the protozoan *T. vaginalis* was first made in the year 1836 by Alfred Donné, a French physician (Powell, 1936). *T. vaginalis* was identified as a motile microorganism present in women who had shown signs of leukorrhea (vaginal discharge which appears to be thick, whitish or yellowish in colour as well as irritation in the genital area) (Harp and Chowdhury, 2011). During the period 1934-1939, an Italian scientist, Procaccini classified and identified the morphological characteristics of *T. vaginalis*. Procaccini identified *T. vaginalis* as being a sexually transmitted disease amongst a group of Italian soldiers who served in the Italian army in Ethiopia. Currently, various studies are being conducted involving

molecular biology and immunologic methods to better understand the pathogenesis of *T. vaginalis* (Harp and Chowdhury, 2011).

1.1.2 Morphology of *T. vaginalis*

Trichomonas vaginalis is known to be one of the most well studied parasites amongst all trichomonads. It is single- celled and oval or pear shaped, i.e. pyriform or amoeboid as shown in Figure 2C (Petrin *et al.*, 1998; Harp and Chowdhury, 2011). However, both the shape and size can differ with the average width and length being 7 µm and 10 µm, respectively (Petrin *et al.*, 1998; Harp and Chowdhury, 2011). Physiochemical conditions can lead to changes in the appearance of the parasite. *T. vaginalis* is a flagellated protozoan having five flagella, four located within the anterior portion whereas the fifth flagellum is integrated within the undulating membrane originating from the blepharoplasty of the parasite as shown in Figure 2A (Petrin *et al.*, 1998; Harp and Chowdhury, 2011).

The cytoskeleton found in the parasite *T. vaginalis* is made up of both actin fibres and tubulins. Researchers have established the presence of different types of tubulin inside a trichomonad cell (Petrin *et al.*, 1998). The actin isolates of *T. vaginalis* are known to migrate slower in comparison to actin isolates from muscles. The location of the nucleus is situated in an anterior portion which is enclosed by a porous nuclear envelope. Another structure found on the parasite *T. vaginalis* is an axostyle which is a small hyaline, rod-like structure. This specific structure overhangs through the posterior end of the parasite enabling the parasite to anchor to the epithelial cells of the vagina resulting in inflammation as shown in Figure 2B (Petrin *et al.*, 1998; Simon, 2018).

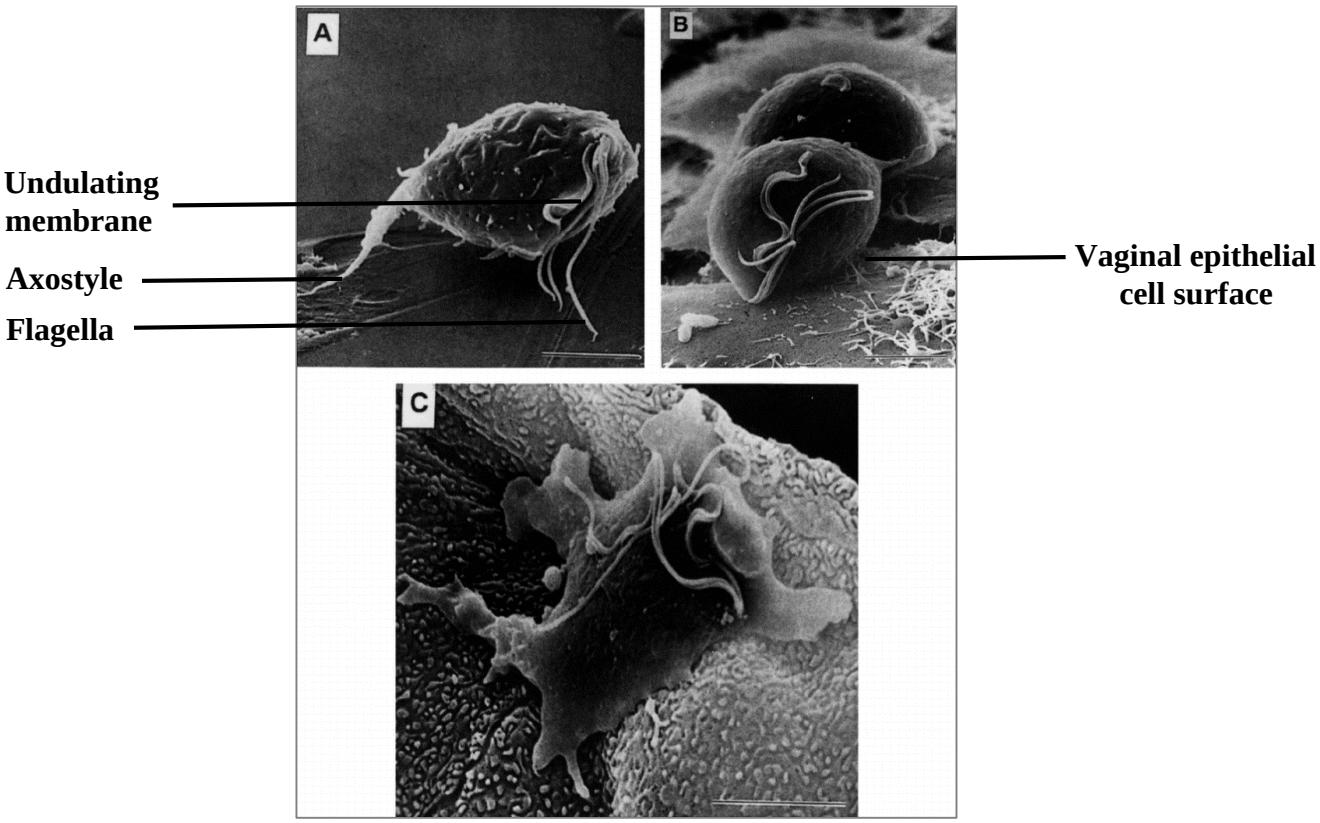

Figure 2: (A) Presence of protozoan *T. vaginalis* in broth culture. The image clearly shows the visibility of the flagella, axostyle and undulating membrane. (B) Protozoan *T. vaginalis* on the surface of a vaginal epithelial cell before amoeboid transformation. (C) *T. vaginalis* amoeboid morphology presence in cell culture. Bars- 5 mm (Petrin *et al*., 1998).

1.1.3 Transmission and life cycle

The life cycle of *T. vaginalis* involves asexual replication via binary fission and mitotic division in humans as the host (Figure 3). This particular parasite colonizes the lower genital tract of women whereas in men it is present in the urethra and prostate (Simon, 2018). *T. vaginalis* does not have a cyst, therefore the flagellate will not be able to survive outside the human body. A moist environment is essential in order for transmission to take place. Sexual transmission is regarded as being the main manner of transmission, even though non-sexual transmission has been reported in previous studies (Crucitti *et al*., 2011). For example, newborn infants can become infected if the mother is already infected with *T. vaginalis*. This occurs when the

parasite enters into the vaginal tract during delivery causing the birth canal to become infected. This results in infants having urinary tract infections, vaginitis and even upper respiratory distress (Danesh *et al*., 1995).

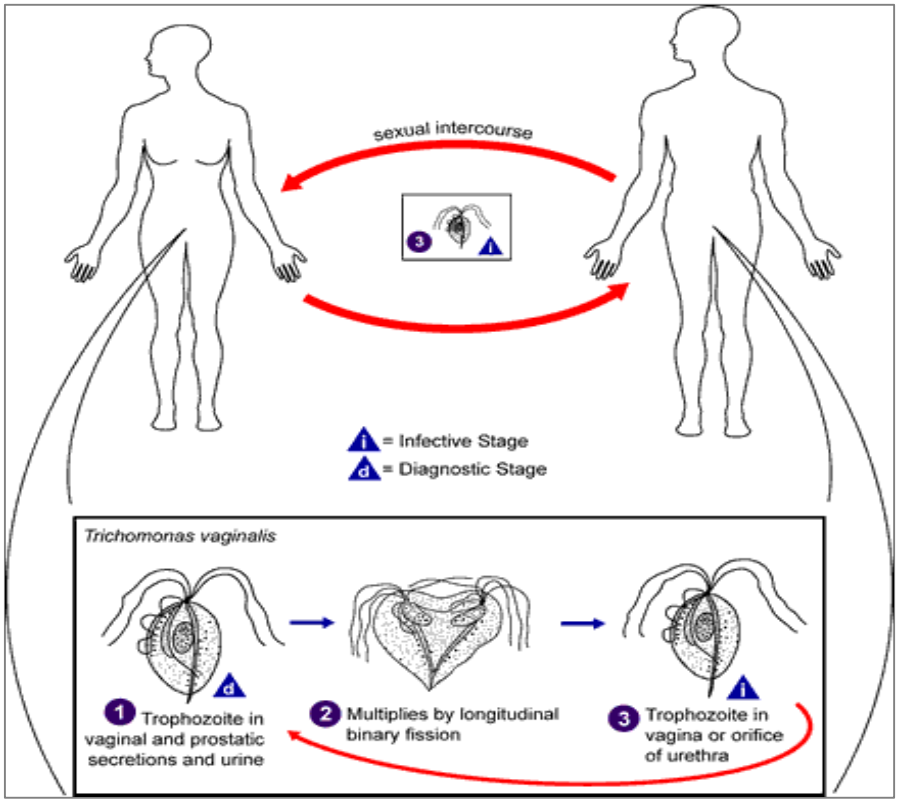

Figure 3: Illustrates both the life cycle and transmission of *T. vaginalis* present in males and females. (1) Presence of the protozoan in the lower genital tract in a female and the prostate and urethra in a male. (2) Replication via binary fission. (3) Transmitted amongst humans via sexual intercourse (Ramos, 2005).

1.1.4 Clinical manifestation and Pathogenesis

There are a number of mechanisms that are involved in the pathogenesis of *T. vaginalis*, such as hemolysis, secretion of soluble factors, and cell-to-cell adhesion, which eventually leads to inflammation and damage to epithelial cells (Gilbert *et al*., 2000). *T. vaginalis* is considered as being a free-living protozoan capable of colonizing the surfaces of mucosal epithelial cells. In

males infected with *T. vaginalis*, the parasite normally persists between 2 weeks to 4 months in the prostrate or urethra (Simon, 2018), whereas in infected females, the parasite can persist up to approximately 5 years in the genital tract (Simon, 2018).

This pathogen has also been detected in individuals who are asymptomatic, hence showing no symptoms of trichomoniasis. Men with trichomoniasis may display the following symptoms; burning sensation during urination or sexual intercourse, discharge from the penis and itching of the penis (Centres for Disease Control (CDC), 2012). Health associated complication in males infected with *T. vaginalis* includes infertility, prostatitis, increased risk of prostate cancer and non-gonococcal urethritis (Conrad *et al*., 2012).

Women infected with trichomoniasis display symptoms including; abnormal vaginal odour ("musty" or ''fishy" vaginal smell), pruritus, lower abdominal pain, purulent vaginal discharge (discharge which is thick and milky white, greenish or yellowish), punctate microhemorrhages referred to as 'strawberry cervix' as well as dyspareunia (genital pain during, before or after sexual intercourse) (Bowden *et al*., 2000; Harp and Chowdhury, 2011; Conrad *et al*., 2012). Studies have shown that approximately 20% of the women who have trichomoniasis infection often develop a foul odour and a foamy discharge (Lehker and Alderete, 2000; Ribeiro *et al*., 2003; Lima *et al*., 2013).

The associated health consequence for women being infected with trichomoniasis, include infertility (Tsevat *et al*., 2017). Generally, women infected with trichomoniasis have a much higher risk of having pelvic inflammatory associated diseases compared to women who are uninfected (Moodley *et al*., 2002).

For pregnant women some of the adverse outcomes of a *T. vaginalis* infection includes having a low-birth weight infant and preterm delivery (Kamal *et al*., 2018). In a study conducted in HIV infected pregnant women from Zimbabwe it was reported that vaginal infections which

includes *T. vaginalis* are critical predictors for vertical transmission of HIV (Gumbo *et al*., 2010).

1.1.5 Treatment of *T. vaginalis* infections

Individuals infected with *T. vaginalis* are commonly treated with a drug referred to as metronidazole (Mz). This drug is a 5-nitroimidazole ($C_3H_3N_3O_2$) drug derived from the *Streptomyces* producing antibiotic azomycin. Studies have shown that metronidazole is highly effective for the treatment of trichomoniasis (Petrin *et al*., 1998). *T. vaginalis* is transmitted mostly sexually, therefore respective partners will also need to be treated.

A metronidazole vaginal gel has also been tested, however, studies have shown that the gel is effective in only approximately half of the reported trichomoniasis cases (Cudmore *et al*., 2004). A study was conducted which compared the treatment for trichomoniasis with vaginal tablets with a low dosage of metronidazole (100 mg) and oral metronidazole (500 mg twice a day) for 7 days. The study showed that treatment with vaginal tablets containing 100 mg of metronidazole resulted in a 64% cure rate (Bouckaert *et al*., 1995; Voorspoels *et al*., 2002; Bouchemal *et al*., 2017).

There have been reports on the emergence of metronidazole resistant *T. vaginalis* (Coelho, 1997; Nyirjesy *et al*., 1998).

A study conducted by Coelho, 1997 reported on a patient who had a trichomoniasis infection with treatment failure to high doses of metronidazole. In another study involving nine patients who were infected with trichomoniasis, four of the nine patients had *T. vaginalis* strains which were metronidazole resistant (Nyirjesy *et al*., 1998).

1.2 Laboratory diagnosis of *T. vaginalis*

1.2.1 Microscopy

Microscopy (wet-mount preparation) still remains a traditional method for the identification of trichomoniasis. Samples are typically obtained from prostatic secretions, urethral secretions and cervical or vaginal samples which are then used to observe motile forms of this particular protozoa, hence flagella movement (Garber, 2005). However, it is important to note that the sensitivity of microscopy ranges from approximately 38% to 82%.

Accurate analysis with microscopy is dependent on a number of factors such as the size of the inoculum, quality of the sample and the observer's experience (Garber, 2005). Non-motile trichomonads are difficult to distinguish from vaginal epithelial cell nuclei. Despite the low cost of this technique, the low levels of sensitivity could lead to a misdiagnosis of *T. vaginalis* (Garber, 2005).

1.2.2 Culture

Over the past 40 years the broth culture technique has been recognized has being the "gold standard'' for *T vaginalis* identification. The size of the inoculum needed for an easy diagnosis is approximately 300 to 500 trichomonads /mL (Petrin *et al*., 1998; Garber, 2005). Diamond's TYI medium is the media of choice for culture. For the identification of *T. vaginalis* in culture an incubation period of between two to seven days is required (Garber, 2005).

A possible limitation with culture is that broth tubes can become easily contaminated with bacteria. However, this can be eliminated by adding antibiotics into the broth to inhibit the growth of the bacteria (Garber, 2005).

In order to improve the adequacy of culture for diagnosis, the development of a plastic envelope technique was established. This self-contained system involves instant examination and culturing. The results obtained from such a system is in fact equivalent to the culture techniques and wet-mount preparation (Petrin *et al*., 1998, Garber, 2005).

1.2.3 Staining

The use of staining techniques was introduced to increase the sensitivity of microscopy (Petrin *et al*., 1998). The types of stains used include; periodic acid Schiff, acridine orange and Fontana and Leishman. Papanicolaou (Pap) staining is popular for trichomoniasis diagnosis, since it is often used in gynaecologic screening to detect cytological abnormalities amongst individuals who have high prevalence rates of STIs. However, a limitation with staining techniques is loss of morphology of *T. vaginalis* as a result of fixation before staining (Petrin *et al*., 1998).

1.2.4 Antibody- Based Techniques

Eight serotypes have been detected in *T. vaginalis*, with the use of immuno-blot analysis, (Garber, 2005). The presence of anti-trichomonal antibodies can be determined via the use of the following techniques; enzyme-linked immunosorbent assay (ELISA), complement fixation, agglutination, indirect hemagglutination, fluorescent antibody and gel diffusion (Petrin *et al*., 1998; Garber, 2005).

The use of monoclonal antibodies can be a promising way of detecting *T. vaginalis* antigens from clinical samples to diagnose trichomoniasis. In a study conducted by Krieger *et al*., 1985 two reactive monoclonal antibodies were able to identify 88 strains of *T. vaginalis* from North American regions. Monoclonal antibodies which were used for the detection of *T. vaginalis* from clinical samples gave similar results to those obtained by wet-mount preparations (Garber, 2005).

1.2.5 DNA- Based Techniques

The diagnosis of *T. vaginalis* using recombinant DNA techniques are often used in clinical laboratories due to improved sensitivity and specificity. DNA based assays such as the BD Affirm VPIII assay, TaqMan based assay and the BD Max ™ Vaginal Panel assay are commercially available assays for the detection of *T. vaginalis* (Dessai *et al.,* 2020; Mabaso *et al.,* 2020). These highly sensitive assays make use of synthetic oligonucleotide probes for detecting *T. vaginalis* from vaginal swabs (Dessai *et al.,* 2020; Mabaso *et al.,* 2020). Dot-blot hybridization is another technique which can be used for the detection of *T. vaginalis*. This technique makes use of a 2.3-kb *T. vaginalis* DNA fragment which acts as a probe and is capable of detecting *T. vaginalis* DNA from vaginal extracts (Petrin *et al*., 1998; Garber, 2005). However, this technique involves the handling of radioactive material, thereby posing a limitation. An alternative technique is *in situ* hybridization which uses a 2.3-kb fluorescence-labelled DNA probe for identifying *T. vaginalis* (Petrin *et al*., 1998; Garber, 2005).

1.3 Genotyping of *T. vaginalis*

Many molecular based techniques have been used in the past in order to distinguish *T. vaginalis* strains. These techniques include; Microsatellite genotyping, Multilocus Sequence Typing (MLST), Polymerase Chain Reaction (PCR) based-hybridization, Random Amplification of Polymorphic DNA (RAPD), PCR-size polymorphism and PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) (Khalili *et al*., 2017).

1.3.1 Microsatellite genotyping

Microsatellites refer to short sequences of nucleotides arranged in tandem repeats with the length generally consisting of two to six base pairs (Conrad *et al*., 2011). Microsatellite genotyping has been considered as an appropriate tool for investigating the diversity of *T.*

vaginalis (Meade and Carlton, 2003). In order to understand the genetic diversity of *T. vaginalis*, a panel of 21 microsatellites and six single copy genes from the *T. vaginalis* genome were recognized and validated. The diversity assessments were conducted in seven laboratory strains of *T. vaginalis* (Conrad *et al*., 2011).

1.3.2 Multilocus Sequence typing (MLST)

Multilocus Sequence typing (MLST) differentiates isolates by making use of internal sequence fragments of about seven housekeeping genes. The genes are amplified by PCR and then sequenced to identify specific alleles (Maiden, 2006; Cornelius *et al*., 2012) (Figure 4). Cornelius *et al*., 2012, conducted a study among 68 *T. vaginalis* isolates. The results obtained with MLST showed the identification of 43 polymorphic nucleotide sites, 51 different alleles as well as 60 sequence types. Van der Veer *et al*., 2016 performed a diversity assessment study on stored samples of men and women who tested positive for *T. vaginalis*. The study results revealed that 7 loci were positively typed using MLST in 81.6% (71/87) of the samples analysed. Samples were separated into genotypes I and II representing the *T. vaginalis* structure in that population group.

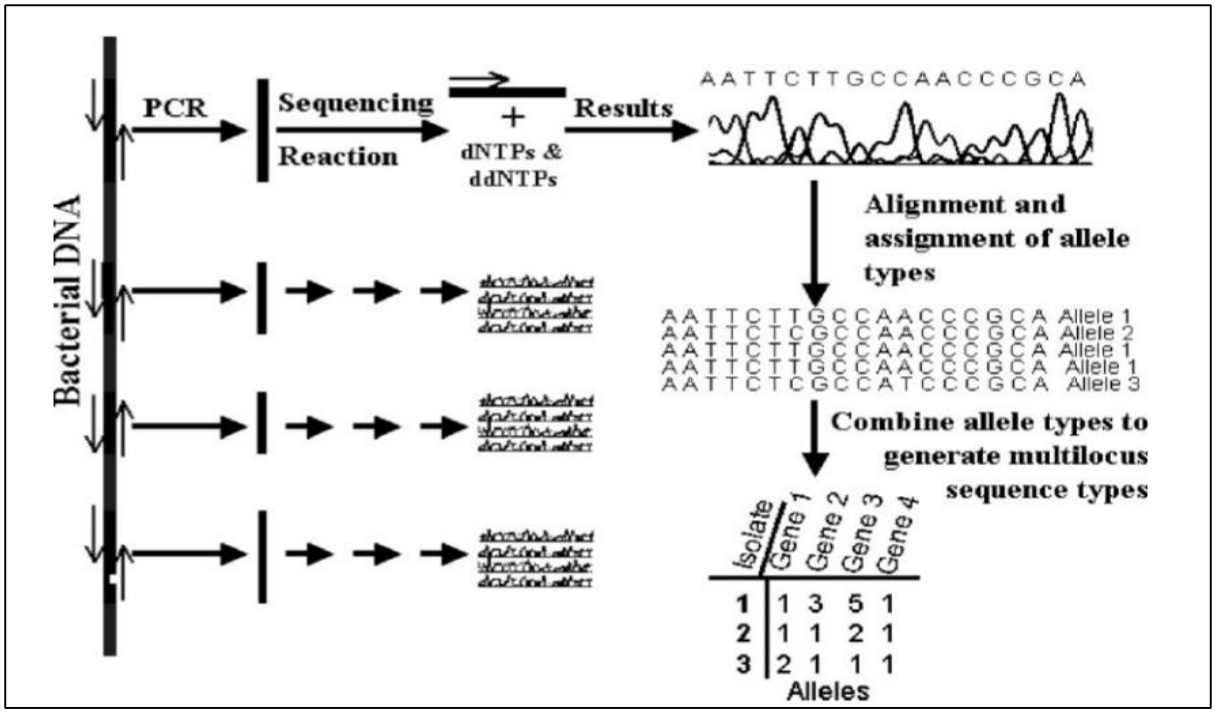

Figure 4: Outline of the MLST procedure. Different gene targets are amplified by making use of the locus-specific primers (represented by the smaller arrows). The genes are sequenced after PCR amplification. The DNA sequences are then matched with other sequences from the MLST database, assigning a name for the allele. The assigned alleles for every locus are then joint resulting in the formation of a Multilocus Sequence type (Singh *et al*., 2006).

1.3.3 Random Amplification of Polymorphic DNA (RAPD)

Random Amplification of Polymorphic DNA (RAPD) genotyping consists of randomly amplifying segments of the target DNA making use of a single primer, which does not show homology to a specific target DNA sequence (Swaminathan and Matar, 1993; Naidoo, 2015). Merdaw *et al*., 2014 conducted a study which used four random primers i.e. OPD1, OPD2, OPD3, and OPD5 in order to determine the genotypic difference amongst *T. vaginalis* isolates obtained from women with and without cervical abnormalities. The results obtained indicated that primers OPD2, OPD3 and OPD5 showed polymorphic bands, whereas primer OPD1 showed no fragment amplification in any of the isolates analysed. This technique can be

compromised by variations in experimental PCR conditions and the presence of contaminating DNA (Meade and Carlton, 2003).

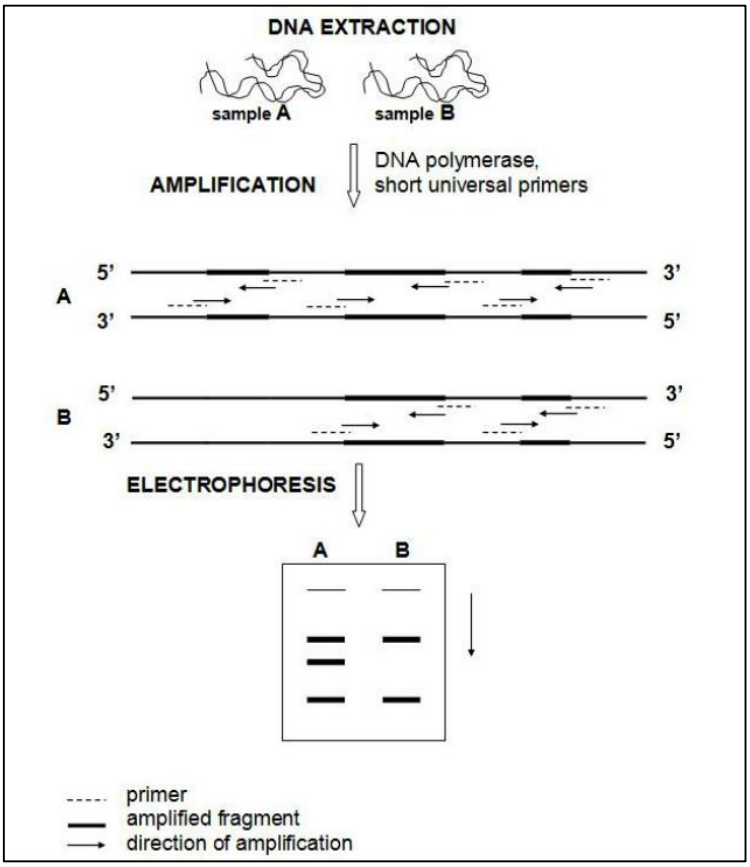

Figure 5: Illustrates the RAPD procedure. Amplification results in multi-fragment PCR patterns, allowing for the screening of polymorphisms in a number of regions in the genome due to the DNA sequence variation at or between the primer-binding sites (Pasqualone, 2013).

1.3.4 PCR– Restriction Fragment Length Polymorphism (PCR-RFLP)

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) involves the amplification of a known DNA sequence, followed by restriction of the resulting amplicons by means of restriction endonuclease enzymes. Gel electrophoresis is then used to determine the differences in the restricted DNA fragment sizes by analysis of banding profiles (Swaminathan and Matar, 1993; Naidoo, 2015).

Genotyping of *T. vaginalis* by PCR-RFLP has been performed by using primers targeting the intergenic spacer (IGS) region of the ribosomal DNA (Simoes-Barbosa *et al*., 2005; Kock *et al*., 2013). A study conducted on 60 clinical isolates of *T. vaginalis*, digested with eight restriction enzymes using IGS-PCR RFLP, showed no genetic variation between isolates (Simoes-Barbosa *et al*., 2005). Similarly, a study performed by Kock *et al*., 2013 in South Africa showed no genetic differences amongst 92 clinical isolates of *T. vaginalis* by IGS-PCR RFLP. However, genetic differences were observed in those same isolates by the RAPD technique (Kock *et al*., 2013). It was therefore suggested that the differentiate capability of PCR-RFLP is dependent specifically on the region that is targeted in the genome.

Crucitti *et al*., 2008 identified different genotypes of *T. vaginalis* based on the digestion of the *actin* gene. The study identified eight *T. vaginalis actin* genotypes amongst 151 isolates obtained from the Democratic Republic of Congo and Zambia. Another study conducted in Africa identified distinct genotypes of *T. vaginalis* based on digestion of the *actin* gene (Masha *et al*., 2017). Studies conducted in Iran, had also reported on the successful assignment of *T. vaginalis* genotypes based on the *actin* gene (Matini *et al*., 2017; Khalili *et al*., 2017; Oliaee *et al*., 2017).

1.4 Rationale

Currently, there is a lack of data on the circulating genotypes of *T. vaginalis* in South African populations, particularly pregnant women. In the present study, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of the *actin* gene was performed in order to identify the different genotypes of *T. vaginalis*. This study provided a snapshot into the molecular epidemiology of *T. vaginalis* in our region which can be used as a foundation for larger epidemiological studies on this pathogen.

1.5 Hypothesis

Due to the relatively high prevalence of *T. vaginalis* in our setting, we hypothesize that a distribution of different genotypes will be present in our study population.

1.6 Aims and objectives

AIM:

The aim of this study was to determine the different genotypes of *T. vaginalis* present in pregnant women from Durban using restriction fragment length analysis.

OBJECTIVES:

1. To detect the presence of *T. vaginalis* by the Polymerase Chain reaction using DNA extracted from vaginal swabs;
2. To determine the genotypes of *T. vaginalis* that are present in this population by restriction fragment length analysis of the *actin* gene; and
3. To link the different genotypes with the clinical data (i.e. symptoms associated with vaginal infections) obtained from the study population.

1.7 Outputs

The Research Article titled *"Genotypic variation in Trichomonas vaginalis detected in South African pregnant women,"* by Rennisha Chetty, Nonkululeko Mabaso and Nathlee S. Abbai has been received and assigned the number 1687427.

Thank you for submitting your work to Infectious Diseases in Obstetrics and Gynecology.

CHAPTER 3
METHODS AND MATERIALS

3.1 Ethics Statement

The current study was approved by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (BREC/00000406/2019) (Appendix I).

3.2 Study design and population

The current study was a retrospective laboratory based study making use of stored DNA extracted from vaginal swabs collected from pregnant women. The swabs were collected through a larger study which was conducted from October 2018-April 2019 (Appendix II). For the larger study, n=362 pregnant women were recruited from the King Edward VIII hospital antenatal clinic in Durban. The women were 18 years and older and willing to provide written informed consent and two self-collected vaginal swabs. At enrolment, all women also provided data on their socio-demographic statuses (age, marital status, level of education), sexual behaviour (age of first sex, cohabitation status, condom use and number of life-time sexual partners) and clinical history (trimester of pregnancy, history of STIs, previous pregnancy history). The data was collected using a structured questionnaire. For this study, de-linked DNA samples were analysed. The parent study contained 362 DNA samples and all these samples were retrieved for analysis in this study.

3.3 Processing and storage of samples from the larger study

Upon collection, one swab was placed immediately in Diamonds media for *T. vaginalis* culture. The second swab (dry swab) was placed in a sterile tube for molecular analysis. The samples were transported at room temperature to the School of Clinical Medicine's Research Laboratory at the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal. The dry swabs were then re-suspended in 2ml of phosphate buffered saline (PBS) and vortexed to

remove the sample material from the swab. After vortexing the swab was discarded and the PBS solution containing the vaginal material was subjected to DNA extraction. For the extraction, the entire 2ml of PBS was used and the DNA was extracted using the PureLink Microbiome DNA Purification Kit (Invitrogen, supplied by ThermoFisher Scientific, United States) according to the manufacturer's instructions. The concentration and purity of the extracted DNA was assessed using the Nanodrop spectrophotometer (ThermoFisher Scientific, United States) prior to storage at -20°C.

3.4 Laboratory procedures

3.4.1 Sample retrieval

The full set of n=362 DNA samples were available for analysis in the current study. All demographic, behavioural and clinical data linked to the stored samples were also available for analysis in this study.

3.4.2 Detection of *T. vaginalis* from stored DNA extracted from vaginal swabs

A total of n=362 vaginal swab DNA samples were tested for the presence of *T. vaginalis*. *T. vaginalis* was detected using the Applied Biosystems™ TaqMan® Assays. Commercial primers and probes (Pr04646256_s1) which target the *alpha tubulin 1* gene of *T. vaginalis* were used. Amplification was performed on the Quant Studio 5 Real-time PCR detection system (ThermoFisher Scientific, United States).

Briefly, each reaction was performed in a final volume of 5 µL which comprised of: 0.5 µL FAM-labelled probe/primer mix, 2.5 µL Fast Start 4x probe master mix (ThermoFisher, Pr04646256_s1), 1.5 µL template DNA and nuclease-free water. We also included non-template control reactions. Amplification was performed under the following conditions: 1 cycle at 95°C for 30 seconds followed by 45 cycles of denaturation at 95°C for 3 seconds and

annealing at 60°C for 30 seconds. Detection of fluorescent products was performed at the end of the annealing period. The raw fluorescent data that included the C_T mean values and gene copy numbers, were automatically generated by the Quant Studio 5 Real-time PCR system software.

3.5 Detection of the *actin* genes from *T. vaginalis*

A conventional nested PCR assay was used for the amplification of the *actin* genes (outer and inner regions) using oligonucleotide primers, published by Espinosa *et al*., 2001 and Khalili *et al*., 2017. The primers used for amplification of inner and outer *actin* genes are shown in Table 1.

Table 1: Description of primers used for the amplification of the *actin* genes in the studied population.

Primer name	**Primer sequence**
Outer actin Forward (Tv8S)	5'-TCTGGAATGGCTGAAGAAGACG-3'
Outer actin Reverse (Tv9R)	5'-CAGGGTACATCGTATTGGTC-3'
Inner actin Forward (Tv10S)	5'-CAGACACTCGTTATCG-3'
Inner actin Reverse (Tv11R)	5'-CGGTGAACGATGGATG-3'

3.6 Amplification of the outer *actin* gene

The amplification reactions were performed with a total volume of 25 µl. The reaction contained 12.5 µl Dream Taq master mix (ThermoFisher Scientific, United States), 9.5 µl distilled water, 0.5 µl of each primer (reverse and forward) and 2 µl of the template DNA. The negative control contained 23 µl of PCR mixture and 2 µl of distilled water. Thereafter, the PCR tubes were placed into the thermal cycler and the following cycling conditions were performed; initial denaturation at 94°C for 5 minutes, followed by 30 cycles each consisting of: denaturation at 94°C for 1 minute, annealing 54°C for 1 minute, elongation 72°C for 1 minute and final elongation at 72°C for 5 minutes.

3.7 Amplification of the inner *actin* gene by nested PCR

The nested amplification reactions were performed in PCR with a total volume of 25 µl. The reaction contained 12.5 µl Dream Taq master mix, 9.5 µl distilled water, 0.5 µl of each primer (reverse and forward) and 2 µl of the outer PCR product. The negative control contained 23 µl of PCR mixture and 2 µl of distilled water. Thereafter, the PCR tubes was placed into the thermal cycler and the following conditions were performed; initial denaturation at 94°C for 5 minutes, thereafter 30 cycles of; denaturation at 94°C for 1 minute, annealing at 45°C for 1 minute, elongation at 72°C for 1 minute and a final elongation at 72°C for 5 minutes.

3.8 Visualization of the PCR amplicons

Agarose gel electrophoresis was performed in order to confirm if the PCR amplification was successful. PCR products were loaded onto a 1% agarose gel which was stained with 4 µl of SYBR safe dye (ThermoFisher Scientific, United States). Each well was loaded with 5 µl of loading dye and 10 µl of the PCR product and was run at a voltage of 100 for 45 minutes, and then viewed using an ultraviolet trans-illuminator (Gene Genius, SYNGENE, United States). The molecular weight marker used for identifying the presence of the bands was the Gene-Ruler 100 bp Plus DNA Ladder, ready-to-use **(**Catalogue number: SM0323).

3.9 Sequence confirmation of the inner *actin* gene

A subset comprising of 10 PCR positive amplicons, i.e. TV184, TV211, TV230, TV232, TV253, TV266, TV270, TV302, TV357, TV358 were sequenced to confirm the presence of the gene prior to the genotyping analysis.

Sanger DNA sequencing was performed on the inner *actin* PCR amplicons. Each amplicon was sequenced in both directions to cover the full length *actin* gene. The sequencing was conducted using the BrilliantDye™ Terminator v3.1 Cycle Sequencing on an ABI3500XL genetic

analyser. The sequencing was performed at Inqaba Biotechnical Industries (Hatfield, Pretoria, South Africa). The ABI sequencing files were edited on CHROMAS (Technelysium, Queensland, Australia). The forward and reverse sequences were aligned using the DNAMAN software (Lynnon Biosoft, California, United States). The identity of the edited sequences was confirmed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST).

3.10 Restriction Fragment Length Polymorphisms (RFLP)

The genotyping of the *actin* gene was performed using the RFLP technique. Restriction enzymes, *HindII*, *MseI*, and *RsaI* were used to generate the banding profiles. The inner *actin* amplicons were digested with the individual enzymes. The digestion mix was made up to a final volume of 20 µl. Each reaction consisted of 0.5 µl enzyme, 2 µl enzyme buffer, 0.2 µl bovine serum albumin (BSA), 7.3 µl distilled water and 10 µl of the PCR product. The digestion reactions were incubated for 4 hours under the following temperature conditions 37° C for both *MseI* and *RsaI* enzymes, and 64°C for the *HindII* restriction enzyme (Khalili *et al*., 2017).

Following incubation, the digests were run on a 2% agarose gel which was stained with 4 µl of SYBR safe dye (ThermoFisher Scientific, United States). Each well was then loaded with 5 µl of loading dye and 20 µl of the digestion mix. The gels were electrophoresed at 80 volts for 2 hours. The enzymes banding patterns and assignment of genotypes based on a composite of the patterns was determined according to Khalili *et al*., 2017. The molecular weight marker used for identifying the presence of the bands for restriction enzyme *MseI* was the Gene-Ruler 100 bp Plus DNA Ladder, ready-to-use **(**Catalogue number: SM0323). Whereas for restriction enzymes *RsaI* and *HindII* Gene-Ruler 50 bp DNA Ladder, ready-to-use (Catalogue number: SM0373) was used.

3.11 Phylogenetic Analysis of *actin* genotypes

Selected samples harbouring different genotypes for the *actin* gene after digestion with the 3 enzymes were selected for further phylogenetic analysis. The amplicons were sequenced in both directions using the Sanger approach. The sequencing was performed on an ABI3500XL genetic analyser at Inqaba Biotechnical Industries (Hatfield, Pretoria, South Africa). The ABI sequencing files were edited on CHROMAS (Technelysium, Queensland, Australia). The forward and reverse sequences were aligned using the DNAMAN software (Lynnon Biosoft, California, United States). The DNA sequences were translated to protein using the freely available software ExPASy (https://web.expasy.org/translate/). The translated sequences together with published Actin protein sequences were aligned using Clustal W (https://www.genome.jp/tools-bin/clustalw). The alignments were performed in order to identify amino acid substitutions. A phylogenetic tree was then constructed from the sequence data using the Molecular Evolutionary Genetics Analysis (MEGA) version 10 software (Arizona, United States).

3.12 Statistical Analysis

Frequency distribution and percentages was used to describe both the demographic and clinical characteristics of the study population. All data was analysed using STATA version 14.0.

CHAPTER 4

RESULTS

4.1 Detection of *T. vaginalis* from DNA extracted from vaginal swabs

The presence of the *alpha tubulin 1* gene was detected in 47/362 (12.98%) swab DNA samples by the real-time PCR TaqMan assay. The quantification cycle (C_q) for the samples that produced positive amplification ranged from 25-35 cycles. All negative no template controls did not produce any amplification. Positive amplification was shown as the curves above the baseline threshold (indicated as a straight red line) (Figure 6).

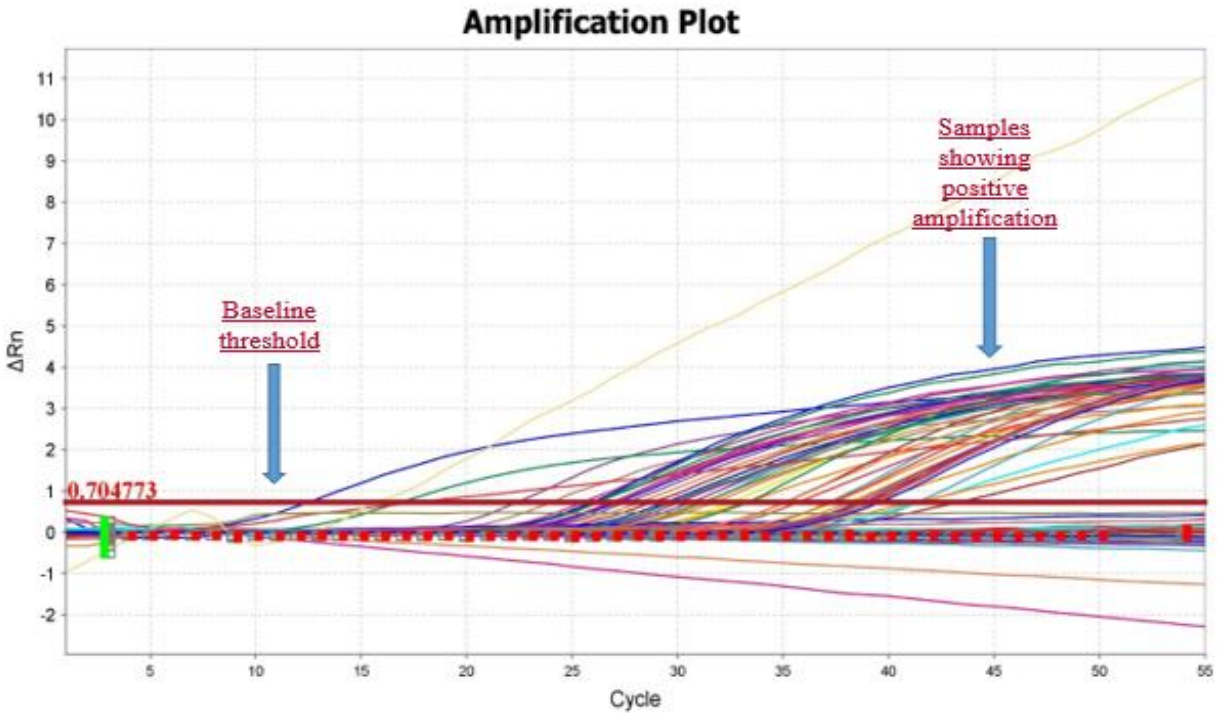

Figure 6: Graph of the amplification plots showing the fluorescent signal from each sample is plotted against cycle number; therefore, representing the accumulation of product over the duration of the real-time PCR experiment. Positive amplification is shown as the curves above the baseline threshold (indicated as a straight red line).

4.2 Baseline characteristics of the women from the parent study

There was a total of 362 pregnant women were screened for *T. vaginalis* (TV) infection. The characteristics of these women according to *T. vaginalis* infection status is summarized in Table 2. A total of 47/362 (13%) women tested positive for *T. vaginalis* infection. A total of 59.6% of the women who tested positive for *T. vaginalis* in this study, did not complain of current abnormal discharge, i.e. were asymptomatic.

Socio- Demographics Status

The baseline characteristics stratified by infection status is summarized in Table 2. For the women who tested *T. vaginalis* positive, majority of them fell in the age groups 20-25 (38.3) and above 30 (38.3). However, age was not significantly associated with infection ($p = 0.172$). There was also no statistical significance with regards to the level of education and employment status ($p = 0.580$; and $p = 0.332$, respectively) for the *T. vaginalis* positive women.

Behavioural factors

There was high prevalence of *T. vaginalis* infection among pregnant women who were not married (91.5%), who had a regular sexual partner (80.9%), who were not living with their partner (57.4%), who had their first sexual encounter between the age of 15 to 20 (70.2%), who had between 2 to 4 life-time sexual partners (66%), who did not know if their partners had other partners (61.7%), and those who reported that they sometimes use condoms (76.6%). However, these behavioral factors were not statistically significant ($p > 0.05$) as shown in Table 2.

Clinical data

Women who were in their third trimester of pregnancy and those who reported no STI symptoms (abnormal vaginal discharge, abnormal vaginal odour and genital itching or sores or warts) in the past three months had a high prevalence of *T. vaginalis* infection (59.6% and 57.4%, respectively), However, these factors were not statistically significant ($p > 0.05$) as indicated in Table 2. There was high prevalence of *T. vaginalis* infection in women that were asymptomatic (no abnormal vaginal discharge at the time of the study enrollment), (prevalence=59.6%, $p = 0.011$).

Table 2: Baseline characteristics of the study population according to *T. vaginalis* infection status.

Variables	***T.vaginalis* Negative (%)**	***T.vaginalis* Positive (%)**	***p*- value**
Age			0.172
15-20	7.9%	4.3%	
21-25	24.4%	38.3%	
26-30	28.3%	19.1%	
>30	39.4%	38.3%	
Education Level			0.580
College, University	26.3%	27.7%	
Didn't attend school	1.9%	0.0%	
High school	69.8%	68.1%	
Primary school	1.9%	4.3%	
Employment			0.332
No	70.8%	63.8%	
Yes	29.2%	36.2%	

Married			0.478
No	87.9%	91.5%	
Yes	12.1%	8.5%	
Regular sexual partner			0.531
No	15.6%	19.1%	
Yes	84.4%	80.9%	
Co-habiting			0.677
No	60.6%	57.4%	
Yes	39.4%	42.6%	
Age at first sex			0.589
<15	1.3%	0.0%	
15-20	75.2%	70.2%	
21-25	20.3%	27.7%	
>25	3.2%	2.1%	
Lifetime sex partners			0.365
1	23.8%	23.4%	
2-4	61.3%	66.0%	
>4	14.9%	10.6%	
Partner has other partners			0.406
Don't know	56.8%	61.7%	
No	23.5%	14.9%	
Yes	19.7%	23.4%	
Condom use			0.646
Always	0.6%	0.0%	
Never	29.2%	21.3%	
Rarely	1.6%	2.1%	
Sometimes	68.6%	76.6%	

Trimester of pregnancy			0.608
1st	13.7%	12.8%	
2nd	34.3%	27.7%	
3rd	52.1%	59.6%	
Current Abnormal Vaginal Discharge			**0.011**
No	76.8%	59.6%	
Yes	23.2%	40.4%	
Previous STI treatment			0.558
No	76.8%	59.6%	
Yes	23.2%	40.4%	
STI symptoms in the past 3 months			0.488
Abnormal Vaginal Discharge	36.2%	34.0%	
Genital itching/sores	14.0%	8.5%	
None	49.8%	57.4%	

4.3 Past clinical symptoms associated with the prevalence of *T. vaginalis* in the antenatal women

For the larger study, the women also provided data on symptoms of vaginal infections experienced in the 3 months prior to enrolment in the study (Figure 7). Table 3, illustrates the percentages of single and combined symptoms experienced. Despite testing positive for *T. vaginalis* in the current study, 57.4% of the women had reported that they had not experienced any past symptoms associated with infection. Abnormal vaginal discharge and genital itching were the most common symptoms experienced in the past (8.5%, respectively). A less common symptom reported was foul smelling vaginal odour (2.1%). Approximately, 6.4% of the women had experienced combined symptoms of abnormal vaginal discharge; genital itching and

vaginal odour. Combined symptoms of abnormal vaginal discharge and vaginal odour (2.1%); abnormal vaginal discharge and genital itching (4.2%); abnormal vaginal discharge, genital itching, vaginal odour, genital ulcers/sores, genital warts (2.1%) were also reported (Table 4).

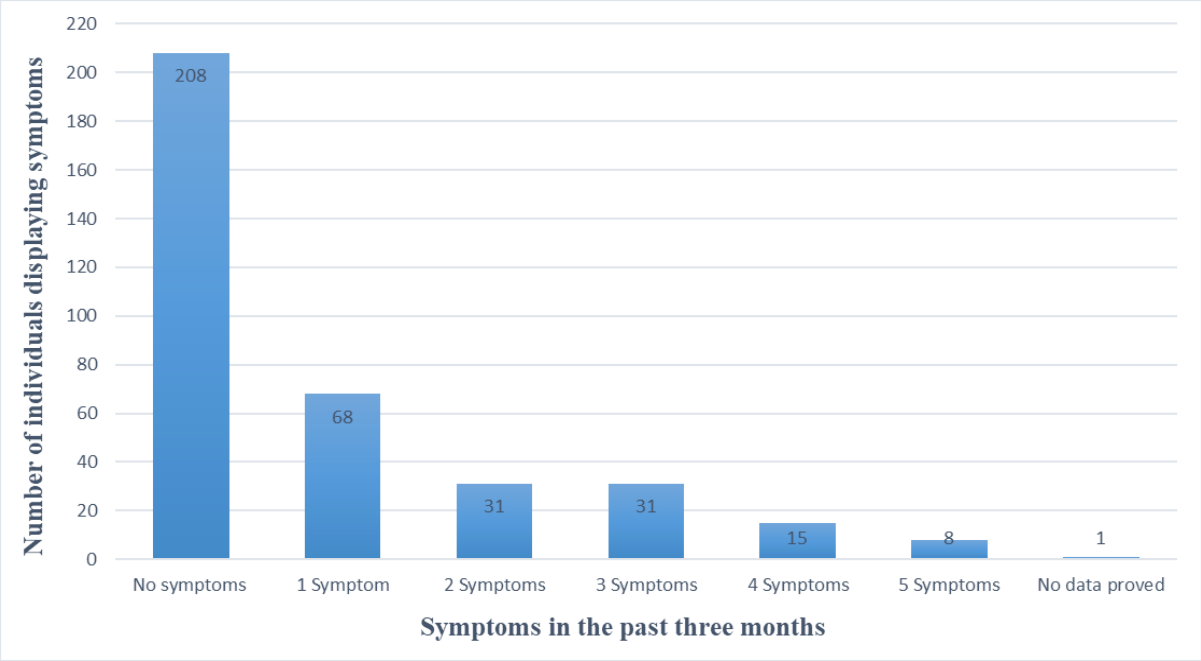

Figure 7: Past number of clinical symptoms associated with vaginal infections reported by the study population (n=362) in the larger study studied.

Table 3: Clinical symptoms displayed by the number of infected women with percentages observed.

Clinical Symptoms Displayed	Number of Women (n=362)	Percentage (%)
No symptoms	208	57.4
AVD	30	8.5
VO	8	2.1
GI	30	8.5
AVD + VO	8	2.1
AVD +GI	15	4.2
VO + GI	8	2.1
AVD + GI + VO	23	6.4
AVD + GI + S/U	8	2.1
AVD + VO + GI + GW	15	4.2
AVD + VO + GI + GW + S/U	8	2.1
No data proved	1	0.3

KEYS: AVD- Abnormal vaginal discharge, GI - Genital Itching, VO- Vaginal Odour, GW- Genital Warts, S/U- Sores/Ulcer

4.4 Results of the amplification reactions

4.4.1 Amplification of the outer *actin* gene of *T. vaginalis*

Amplification of the outer *actin* gene by conventional PCR yielded 16 positives out of the 47 samples tested. The majority of the samples (23/31) that did not test positive in the initial PCR showed either low DNA concentration or purity ratios (Appendix II). For the low concentration samples, the reactions were repeated with an increased amount of DNA, however, the adjustment still did not result in successful amplification of the outer *actin* gene. For lower purity samples, the suggestion of potential sample inhibitors that could have negatively affected the PCR reaction was not found to hold true, since the samples were amplifiable for other genes such as the *Mycoplasma hominis* 16S *rRNA* that were not part of this study (data not shown).

For the 16 samples that produced a positive amplicon the expected fragment size of 1300bp was observed after agarose gel electrophoresis (Figure 8). No amplification was noted for the no template control (negative control- lane 2, Figure 8). A sub-set of 10 samples is shown in Figure 8.

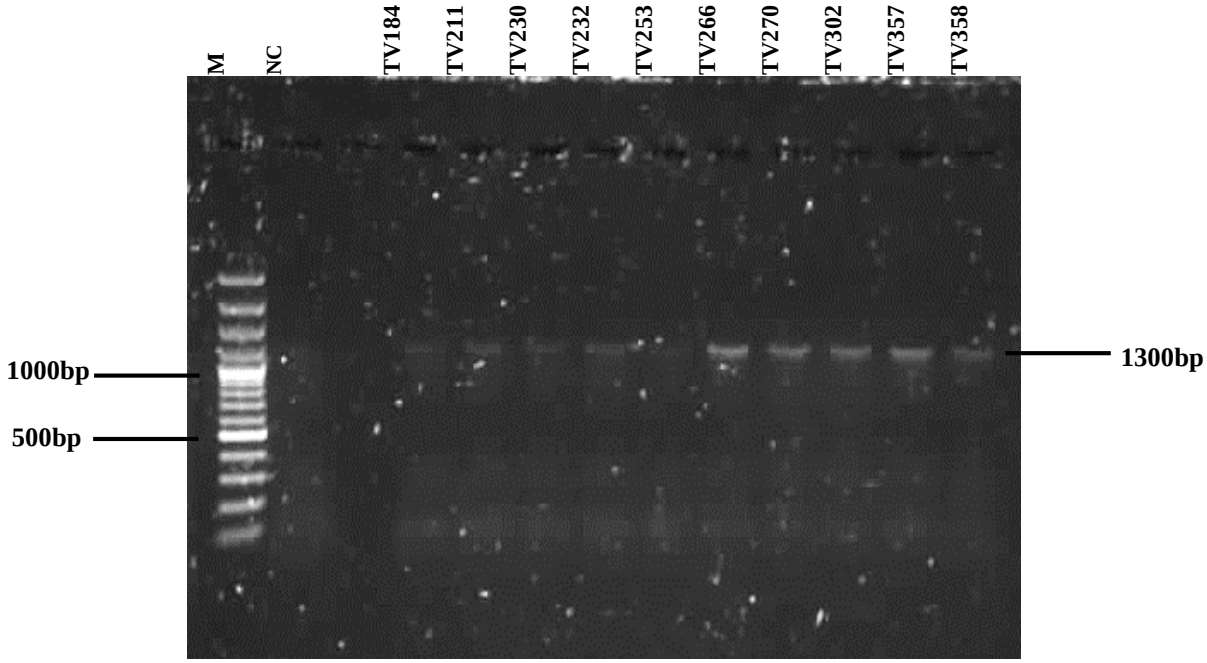

Figure 8: Agarose gel showing positive amplicons generated for the outer *actin g*ene. The expected fragment size of 1300bp was observed. M: 100bp DNA molecular ladder (ThermoFisher Scientific), NC: negative control (no template DNA added) and Lane 3-12: clinical samples amplified clinical samples.

4.4.2 Amplification of the inner *actin* gene of *T. vaginalis* by nested PCR

The 16 positive amplicons generated for the outer *actin* gene were used as a template for the PCR reactions to amplify the inner *actin* gene. The inner *actin* gene was amplified in all 16 samples. The expected fragment size of 1100bp was observed after agarose gel electrophoresis (Figure 9). The fragments were of a good quality (single band of high intensity) for further restriction fragment analysis.

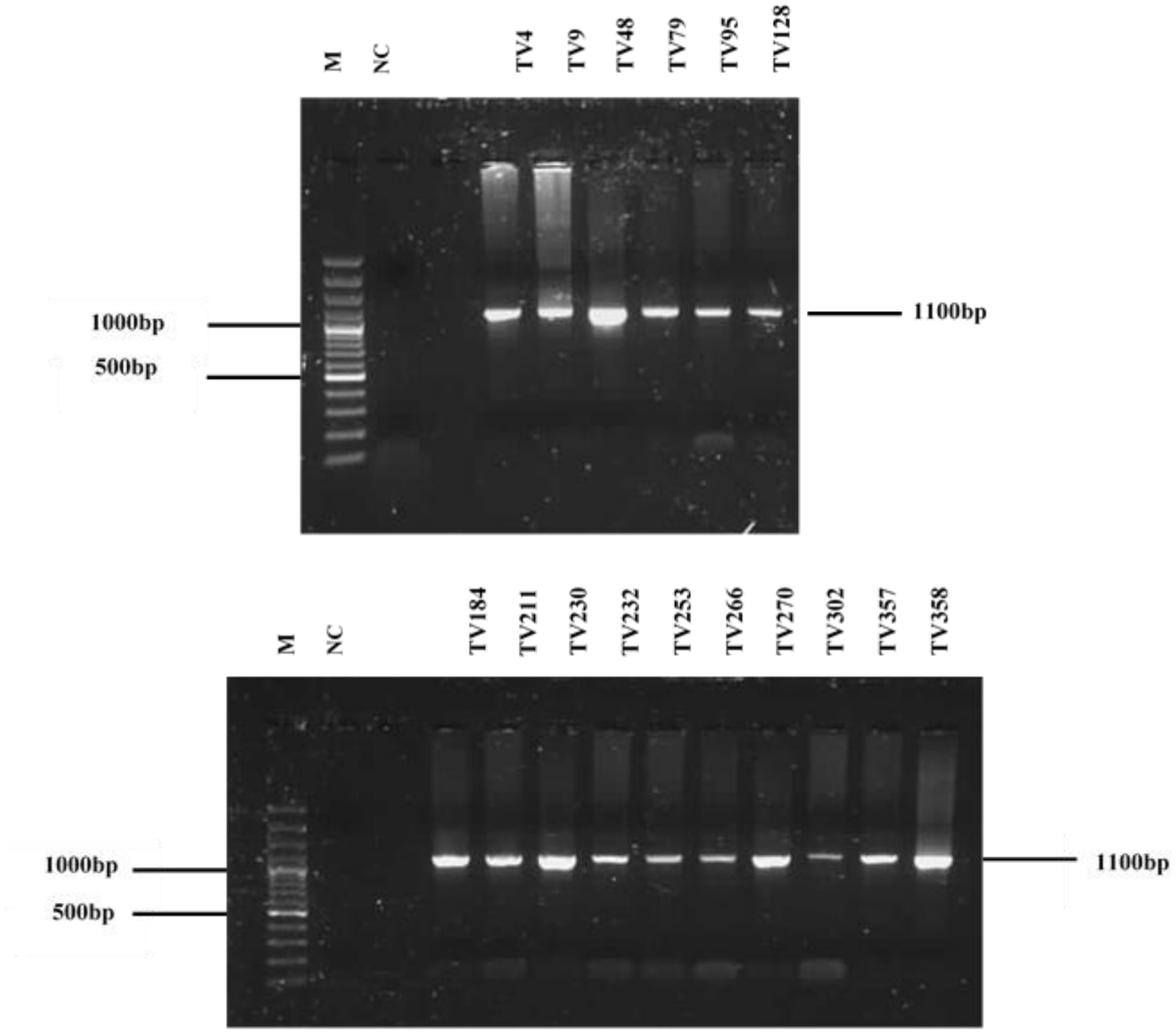

Figure 9: Agarose gel showing positive amplicons generated for the inner *actin g*ene. The expected fragment size of 1100bp was observed. M: 100bp DNA molecular ladder (ThermoFisher Scientific), NC: negative control (no template DNA added) and Amplified clinical samples. A product size of 1100bp indicative of the inner *actin* gene was present in all 16 samples tested.

4.5 Sequence confirmation of the *actin* gene prior to genotyping analysis

Sanger sequencing confirmed that the amplicons generated with the *actin* primers were the *actin* gene from *T. vaginalis.* The DNA sequencing hits of the *actin* gene showed identity (99%) to *T. vaginalis* isolate 19 *actin* gene (MF350343.1) and *T. vaginalis* strain ATCC 30240 *actin* gene (99%) (EU076579.1) (Table 4).

Table 4: DNA sequencing hits according to NCBI BLAST confirming the presence of the *actin* gene amplified from clinical isolates testing positive for *T. vaginalis***.**

Query sample	BLAST HIT	% Identity	Accession number
TV184	*Trichomonas vaginalis* isolate 19 actin gene, partial cds	99%	MF350343.1
TV211	*Trichomonas vaginalis* isolate 19 actin gene, partial cds	99%	MF350343.1
TV230	*Trichomonas vaginalis* isolate 19 actin gene, partial cds	99%	MF350343.1
TV232	*Trichomonas vaginalis* isolate 19 actin gene, partial cds	98%	MF350343.1
TV253	*Trichomonas vaginalis* isolate 19 actin gene, partial cds	98%	MF350343.1
TV266	Trichomonas vaginalis isolate 16 actin gene, partial cds	98	MF350337.1
TV270	*Trichomonas vaginalis* isolate 19 actin gene, partial cds	98%	MF350343.1
TV302	*Trichomonas vaginalis* isolate 19 actin gene, partial cds	99%	MF350343.1

TV357	*Trichomonas vaginalis* isolate 19 actin gene, partial cds	99%	MF350343.1
TV358	Trichomonas vaginalis strain ATCC 30240 actin gene, partial cds	99%	EU076579.1

4.6 Genotyping analysis

4.6.1 *HindII* profile

All samples produced a banding pattern for *HindII*. A consistent banding profile was observed across all samples (Figure 10). Numerous bands were observed for this digest which had not been previously published (Khalili *et al.*, 2017). Since all the samples produced the same banding profile, one sample (TV266) was selected (based on having a bright banding pattern, Figure 10) for analysis using the freely available Restriction mapper tool ((http://www.restrictionmapper.org/cgi-bin/sitefind3.pl). According to Restriction mapper, only four fragments should have been yielded at positions, 60bp, 200bp, 386bp and 426bp (Table 5). These four fragments were shown to be present on the gel (Figure 10). The additional bands observed could have been due to star activity by the enzyme (i.e. cutting at non-specific recognition sites due to prolonged incubation period or too high enzyme concentration). Based on the identical banding pattern across all samples, the samples were assigned the same pattern code i.e. Pattern 2 (Table 6).

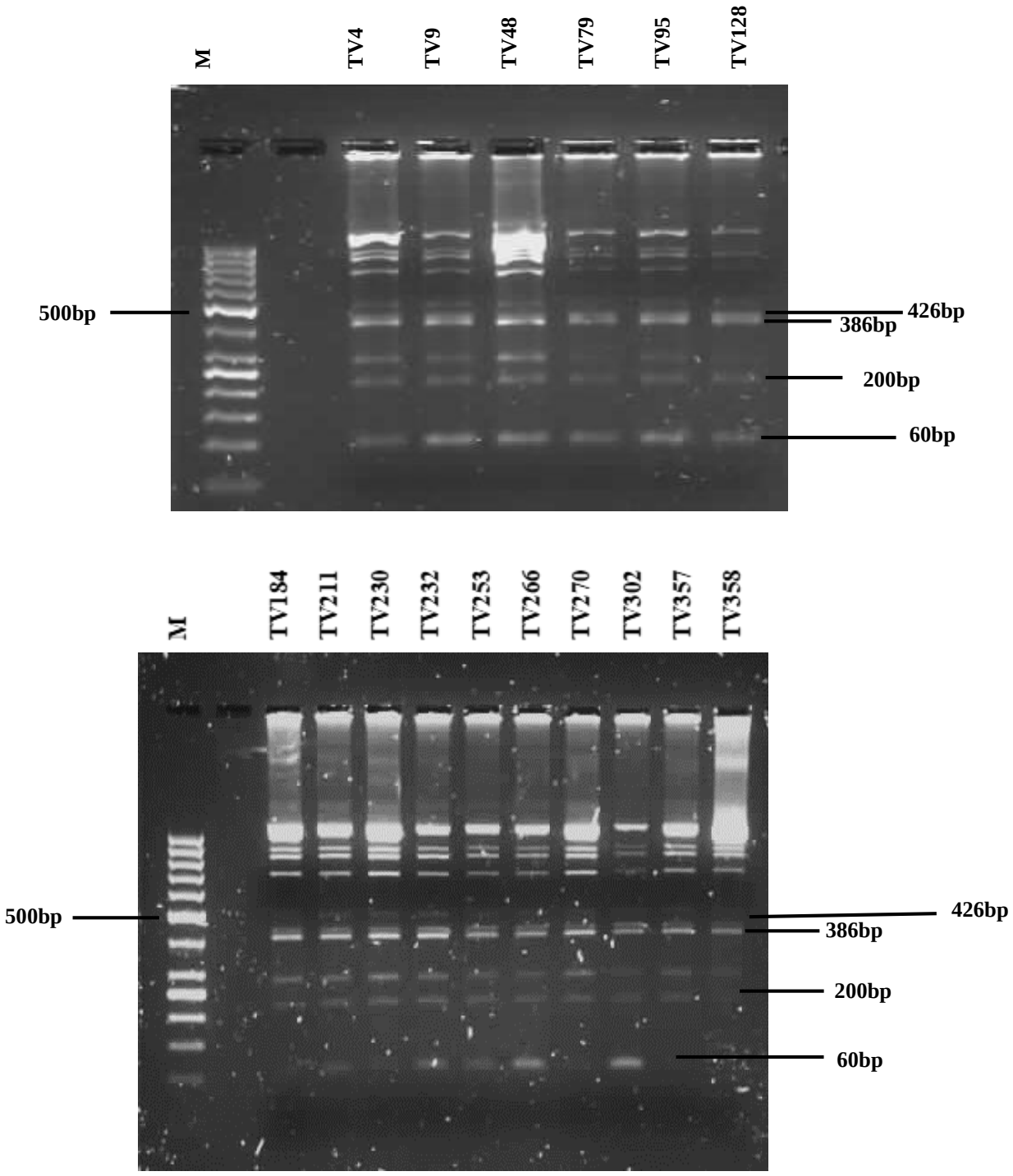

Figure 10: *HindII* RFLP pattern of the digested *actin* gene amplicon resolved on a 1.5% agarose gel. M: O'GeneRuler 50bp DNA Ladder (ThermoFisher Scientific) and banding profiles of the clinical samples. Size fragments of 60bp, 200bp, 386bp and 426bp were observed for the respective samples.

Table 5: Virtual digest of sample TV266 with the Restriction Mapper tool. **(http://www.restrictionmapper.org/cgi-bin/sitefind3.pl)**

Length	5' Enzyme	5' Base	3' Enzyme	3' Base	Sequence
426	*HindII*	261	*HindII*	686	GACCCAACAG AGCACCCAGT TCTTCTTACA GAAGCCCCAC TCAACCCAAAGGCTAACCGT GAGAAAATGA TCTCCCTCAT GTTCGACACATTCAATGTCCCATCCTTCTA TGTCGGCATC CAGGCTGTTCTTTCCCTCTA CTCCTCTGGCCGTACAACAGGTATCGTTTT CGATGCTGGT GATGGTGTTT CCCACACAGTTCCAATTTAC GAAGGCTACT CCCTTCCACA CGCCATCATG AGACTTAACCTCGCTGGCCG TGATCTCACA GCCTGGATGG TCAAGCTTCT CACAGAGCGTGGCAATGCTT TCAACACAAC AGCCGAAAAG GAAATCGTTC GTGACATCAAGGAGAAGCTT TGCTATGTCG CCCTCGACTT CGATGCTGAA ATGGAGAAGGCCGCTACAGA CTCCTCCATC AACGTC
386	*HindII*	687	none	1072	AACTACACAC TTCCAGATGG CAACGTCATC ACAATCGGCA ATGAGCGCTTCCGCTGCCCA GAAATGCTCT TCAAGCCATA CTTCGATGGT ATGGAATACGATGGTATCGA CAAGACACTC TTCGACTCCA TCATGAAGTG CGATATCGATGTTCGTAAGG ATCTCTACGC TAACATCGTT CTTTCTGGTG GCACAACAATGTTCCAAGGG CATCGCCGAA CGTCTTGACA AGGAAATCAC AGCTCTTGCTCCACCAACAA TGAAGGTCAA GATCGTCGCC CCAGAAGAGC GTAAGTACGCCGTTTGGGTC GGTGGCTCCA TCCTTGCTTC CCTCGCTACA TCCCACAGATGGGTATCACA AGGAGGAATA CGACGAGGCT GGTCTC
200	none	1	*HindII*	200	ACGATACGCT CTGGTATGTG CAAGACGGCT TCTCTGGCGA TGAAGCCCCACGCTCTGTTT TTCCCATCCG TTGTTGGCCG ATCCAAAAGT ACAAACAACAATTAGTTGGT GGCAACGCCA AGGATGTCTT CGTCGGTGAT GAAGCTTGCTCCAAGGCTGG TGTCCTCATC CTCAAGTACC CAATTGAACA CGGTATCGTC
60	*HindII*	201	*HindII*	260	AACAACTGGG ATGATATGGA AAAGATCTGG CACCACACAT TCTACAACGAACTTCGTGTT

4.6.2 *RsaI* profile

All samples produced banding patterns for *RsaI*. Three different banding patterns were observed for *RsaI* (Figure 11). Of the 16 samples digested, 13/16 (81.25%) of the samples produced four fragment sizes at positions; 106bp, 190bp, 236bp and 568bp. This group of samples were assigned the code, Pattern 1. Two of the 16 samples (12.50%) produced three fragment sizes at positions; 106bp, 236bp and 568bp and the one sample (6.25%) produced

four fragments at positions; 106bp, 190bp, 236bp and 452bp. The groups were assigned the codes, Pattern H and I, respectively (Table 6). The size of the digestion fragments obtained corresponded to previous studies which reported on genotyping of the *actin* gene by *RsaI* digestion.

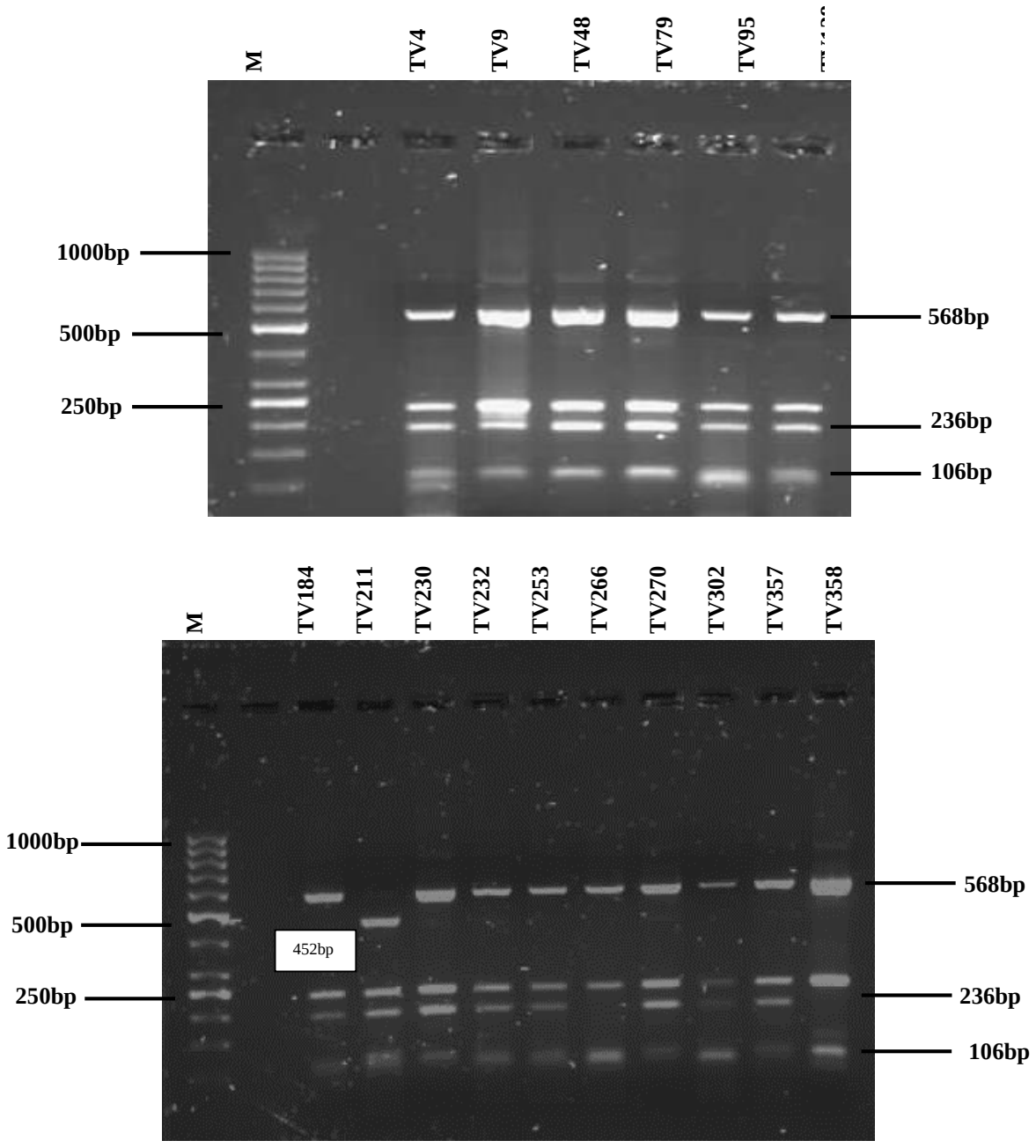

Figure 11: *Rsa*I RFLP pattern of the digested *actin* gene amplicon resolved on a 1.5% agarose gel. O'GeneRuler 50bp DNA Ladder (ThermoFisher Scientific) and banding profiles of the clinical samples. Fragment sizes of 106bp, 236bp, 452bp and 568bp were observed.

4.6.3 *MseI* profile

For the *MseI* reactions, one sample did not produce a banding pattern despite numerous adjustments such as increased incubation time and increased amounts of template DNA. The sample produced a very faint uncut band. For the remaining 15/16 (93.75%) of the samples, an identical banding profile was observed for all the samples. Bands at positions 519bp and 581bp were observed (Figure 12). All of the samples producing a digestion pattern were assigned the code, Pattern 1 (Table 6).

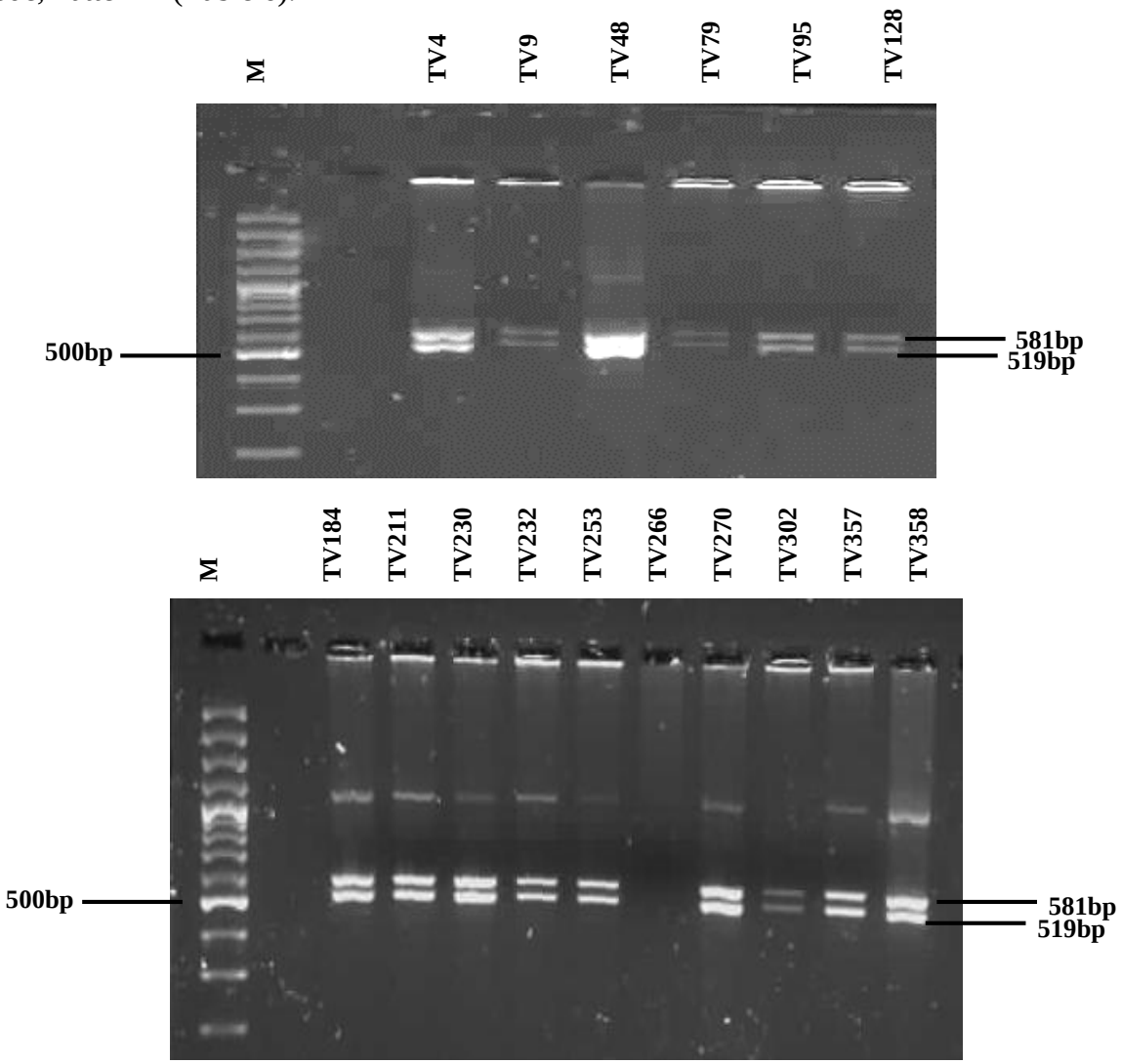

Figure 12: *Mse*I RFLP pattern of the digested *actin* gene amplicon resolved on a 1.5% agarose gel. M: 100bp DNA ladder (Thermofisher Scientific) and banding profiles of the clinical samples. Fragment sizes of 519bp and 581bp were observed.

4.6.4 Frequency of genotypes

According to the combined pattern codes of all three enzymes, genotype G was the most frequent genotype in our study population. A total of 13/16 (81.25%) of the samples contained this genotype. One sample (TV358) harboured genotype H. Similarly, one sample (TV211) harboured genotype I (6.25%). There was one sample (TV266) that could not be assigned a genotype since it lacked a banding profile for *MseI*, a pattern code could therefore not be determined. This sample was therefore excluded from further sequence analysis (Table 6).

Table 6: Fragment sizes obtained after digestion of the *actin* gene with the restriction enzymes *HindII*, *MseI* and *RsaI* as well as the assignment of the *T. vaginalis* genotypes based on combining the patterns across the three enzyme profiles.

Sample name	*HindII* fragment sizes	*HindII* pattern	*MseI* fragment sizes	*MseI* pattern	*RsaI* fragment sizes	*RsaI* pattern	Genotype
TV4	60, 200 386,426	2	519,581	1	106,190,236,568	1	G
TV9	60, 200 386,426	2	519, 581	1	106,190,236,568	1	G
TV48	60, 200 386,426	2	519, 581	1	106,190,236,568	1	G
TV79	60, 200 386,426	2	519, 581	1	106,190,236,568	1	G
TV95	60, 200 386,426	2	519, 581	1	106,190,236,568	1	G
TV128	60, 200 386,426	2	519, 581	1	106,190,236,568	1	G
TV184	60, 200 386,426	2	519, 581	1	106,190,236,568	1	G
TV211	60, 200 386,426	2	519, 581	1	106,190,236,452	3	I
TV230	60, 200 386,426	2	519, 581	1	106,190,236,568	1	G
TV232	60, 200 386,426	2	519, 581	1	106,190,236,568	1	G
TV253	60, 200 386,426	2	519, 581	1	106,190,236,568	1	G
TV266#	60, 200 386,426	2	-*		106,236,568	2	Could not be assigned
TV270	60, 200 386,426	2	519, 581	1	106,190,236,568	1	G
TV302	60, 200 386,426	2	519, 581	1	106,190,236,568	1	G
TV357	60, 200 386,426	2	519, 581	1	106,190,236,568	1	G
TV358	60, 200 386,426	2	519, 581	1	106,236,568	2	H

* symbol indicates that no banding pattern was observed.

genotype could not be assigned due to no banding pattern for *MseI*

4.7 Phylogenetic analysis of the *actin* open reading frame

Translated nucleotide sequences obtained for the *actin* gene from nine clinical samples were edited and aligned with published sequences for genotypes G and H. There were no published sequences for genotype I to include in the comparative analysis. According to the multiple sequence alignment of the study samples with published *T. vaginalis actin* sequences, a total of four different single nucleotide changes in the open reading frame (ORF) of the *actin* gene were observed (Figure 13). Sample TV358 (genotype H) contained a single amino acid subsititution from Glutamine (Q) to Lysine (K). Sample TV184 (genotype G) also contained a single amino acid subsititution from Glutamic acid (E) to Arginine (R). Sample TV357 (genotype G) contained two amino acid substitutions. The first change was the replacement of Leucine (L) with Arginine (R) and the second change was the replacement of Glycine (G) with Aspartic acid (D). The change from G→D was also observed for a published *T. vaginalis ATCC* genotype G strain (EU076578) (Figure 13).

```
TV270           -------HPSLSRPKYKQQLVGGNAKDVFVGDEACSKAGVLILKYPIEHGIVNNWDDMEK
TV302           -------HPLFSRPKYKQQLVGGNAKDVFVGDEACSKAGVLILKYPIEHGIVNNWDDMEK
TV230           ------------------------------------------------------MEK
TV232           ------------------------------------------------------MEK
TV211           ------------------------------------------------------MEK
TV358           -------IRLLARPKYKQQLVGGNAKDVFVGDEACSKAGVLILKYPIEHGIVNNWDDMEK
KF747377        EAPRSVFPSVVGRPKYKQQLVGGNAKDVFVGDEACSKAGVLILKYPIEHGIVNNWDDMEK
EU076579        EAPRSVFPSVVGRPKYKQQLVGGNAKDVFVGDEACSKAGVLILKYPIEHGIVNNWDDMEK
EU076581        EAPRSVFPSVVGRPKYKQQLVGGNAKDVFVGDEACSKAGVLILKYPIEHGIVNNWDDMEK
EU076578        EAPRSVFPSVVGRPKYKQQLVGGNAKDVFVGDEACSKAGVLILKYPIEHGIVNNWDDMEK
TV357           EAPRSVFPSVVGRPKYKQQLVGGNAKDVFVGDEACSKAGVLILKYPIEHGIVNNWDDMEK
TV184           ------------------------------------------------------MEK
TV253           ------------------------------------------------------MEK
                                                                       ***

TV270           IWHHTFYNELRVDPTEHPVLLTEAPLNPKANREKMISLMFDTFNVPSFYVGIQAVLSLYS
TV302           IWHHTFYNELRVDPTEHPVLLTEAPLNPKANREKMISLMFDTFNVPSFYVGIQAVLSLYS
TV230           IWHHTFYNELRVDPTEHPVLLTEAPLNPKANREKMISLMFDTFNVPSFYVGIQAVLSLYS
TV232           IWHHTFYNELRVDPTEHPVLLTEAPLNPKANREKMISLMFDTFNVPSFYVGIQAVLSLYS
TV211           IWHHTFYNELRVDPTEHPVLLTEAPLNPKANREKMISLMFDTFNVPSFYVGIQAVLSLYS
TV358           IWHHTFYNELRVDPTEHPVLLTEAPLNPKANREKMISLMFDTFNVPSFYVGIQAVLSLYS
KF747377        IWHHTFYNELRVDPTEHPVLLTEAPLNPKANREKMISLMFDTFNVPSFYVGIQAVLSLYS
EU076579        IWHHTFYNELRVDPTEHPVLLTEAPLNPKANREKMISLMFDTFNVPSFYVGIQAVLSLYS
EU076581        IWHHTFYNELRVDPTEHPVLLTEAPLNPKANREKMISLMFDTFNVPSFYVGIQAVLSLYS
EU076578        IWHHTFYNELRVDPTEHPVLLTEAPLNPKANREKMISLMFDTFNVPSFYVGIQAVLSLYS
TV357           IWHHTFYNELRVDPTEHPVLLTEAPLNPKANREKMISLMFDTFNVPSFYVGIQAVLSLYS
TV184           IWHHTFYNELRVDPTEHPVLLTEAPLNPKANREKMISLMFDTFNVPSFYVGIQAVLSLYS
TV253           IWHHTFYNELRVDPTEHPVLLTEAPLNPKANREKMISLMFDTFNVPSFYVGIQAVLSLYS
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TV270           SGRTTGIVFDAGDGVSHTVPIYEGYSLPHAIMRLNLAGRDLTAWMVKLLTERGNAFNTTA
TV302           SGRTTGIVFDAGDGVSHTVPIYEGYSLPHAIMRLNLAGRDLTAWMVKLLTERGNAFNTTA
TV230           SGRTTGIVFDAGDGVSHTVPIYEGYSLPHAIMRLNLAGRDLTAWMVKLLTERGNAFNTTA
TV232           SGRTTGIVFDAGDGVSHTVPIYEGYSLPHAIMRLNLAGRDLTAWMVKLLTERGNAFNTTA
TV211           SGRTTGIVFDAGDGVSHTVPIYEGYSLPHAIMRLNLAGRDLTAWMVKLLTERGNAFNTTA
TV358           SGRTTGIVFDAGDGVSHTVPIYEGYSLPHAIMRLNLAGRDLTAWMVKLLTERGNAFNTTA
KF747377        SGRTTGIVFDAGDGVSHTVPIYEGYSLPHAIMRLNLAGRDLTAWMVKLLTERGNAFNTTA
EU076579        SGRTTGIVFDAGDGVSHTVPIYEGYSLPHAIMRLNLAGRDLTAWMVKLLTERGNAFNTTA
EU076581        SGRTTGIVFDAGDGVSHTVPIYEGYSLPHAIMRLNLAGRDLTAWMVKLLTERGNAFNTTA
EU076578        SGRTTGIVFDAGDGVSHTVPIYEGYSLPHAIMRLNLAGRDLTAWMVKLLTERDNAFNTTA
TV357           SGRTTGIVFDAGDGVSHTVPIYEGYSLPHAIMRRNLAGRDLTAWMVKLLTERDNAFNTTA
TV184           SGRTTGIVFDAGDGVSHTVPIYEGYSLPHAIMRLNLAGRDLTAWMVKLLTERGNAFNTTA
TV253           SGRTTGIVFDAGDGVSHTVPIYEGYSLPHAIMRLNLAGRDLTAWMVKLLTERGNAFNTTA
                ********************************* ******************.*******

TV270           EKEIVRDIKEKLCYVALDFDAEMEKAATDSSINVNYTLPDGNVITIGNERFRCPEMLFKP
TV302           EKEIVRDIKEKLCYVALDFDAEMEKAATDSSINVNYTLPDGNVITIGNERFRCPEMLFKP
TV230           EKEIVRDIKEKLCYVALDFDAEMEKAATDSSINVNYTLPDGNVITIGNERFRCPEMLFKP
TV232           EKEIVRDIKEKLCYVALDFDAEMEKAATDSSINVNYTLPDGNVITIGNERFRCPEMLFKP
TV211           EKEIVRDIKEKLCYVALDFDAEMEKAATDSSINVNYTLPDGNVITIGNERFRCPEMLFKP
TV358           EKEIVRDIKEKLCYVALDFDAEMEKAATDSSINVNYTLPDGNVITIGNERFRCPEMLFKP
KF747377        EKEIVRDIKEKLCYVALDFDAEMEKAATDSSINVNYTLPDGNVITIGNERFRCPEMLFKP
EU076579        EKEIVRDIKEKLCYVALDFDAEMEKAATDSSINVNYTLPDGNVITIGNERFRCPEMLFKP
EU076581        EKEIVRDIKEKLCYVALDFDAEMEKAATDSSINVNYTLPDGNVITIGNERFRCPEMLFKP
EU076578        EKEIVRDIKEKLCYVALDFDAEMEKAATDSSINVNYTLPDGNVITIGNERFRCPEMLFKP
TV357           EKEIVRDIKEKLCYVALDFDAEMEKAATDSSINVNYTLPDGNVITIGNERFRCPEMLFKP
TV184           EKEIVRDIKEKLCYVALDFDAEMEKAATDSSINVNYTLPDGNVITIGNERFRCPEMLFKP
TV253           EKEIVRDIKEKLCYVALDFDAEMEKAATDSSINVNYTLPDGNVITIGNERFRCPEMLFKP
                ************************************************************

TV270           YFDGMEYDGIDKTLFDSIMKCDIDVRKDLYANIVLSGGTTMFQGIAERLDKEITALAPPT
TV302           YFDGMEYDGIDKTLFDSIMKCDIDVRKDLYANIVLSGGTTMFQGIAERLDKEITALAPPT
TV230           YFDGMEYDGIDKTLFDSIMKCDIDVRKDLYANIVLSGGTTMFQGIAERLDKEITALAPPT
TV232           YFDGMEYDGIDKTLFDSIMKCDIDVRKDLYANIVLSGGTTMFQGIAERLDKEITALAPPT
TV211           YFDGMEYDGIDKTLFDSIMKCDIDVRKDLYANIVLSGGTTMFQGIAERLDKEITALAPPT
TV358           YFDGMEYDGIDKTLFDSIMKCDIDVRKDLYANIVLSGGTTMFKGIAERLDKEITALAPPT
KF747377        YFDGMEYDGIDKTLFDSIMKCDIDVRKDLYANIVLSGGTTMFQGIAERLDKEITALAPPT
EU076579        YFDGMEYDGIDKTLFDSIMKCDIDVRKDLYANIVLSGGTTMFQGIAERLDKEITALAPPT
EU076581        YFDGMEYDGIDKTLFDSIMKCDIDVRKDLYANIVLSGGTTMFQGIAERLDKEITALAPPT
EU076578        YFDGMEYDGIDKTLFDSIMKCDIDVRKDLYANIVLSGGTTMFQGIAERLDKEITALAPPT
TV357           YFDGMEYDGIDKTLFDSIMKCDIDVRKDLYANIVLSGGTTMFQGIAERLDKEITALAPPT
TV184           YFDGMEYDGIDKTLFDSIMKCDIDVRKDLYANIVLSGGTTMFQGHRRTS-----------
TV253           YFDGMEYDGIDKTLFDSIMKCDIDVRKDLYANIVLSGGTTMFQGIAERLDKEITALAPPT
                ****************************************:*  .
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Figure 13: Multiple sequence alignment of the actin ORF from clinical samples and published genotypes using Clustal W (GenomeNet). A total of 4 different single nucleotide changes in the open reading frame (ORF) of the *actin* gene were detected for the clinical samples (changes highlighted in yellow). A single change in one of the published sequences was also noted (highlighted in green).

The phylogenetic analysis of the *actin* gene ORF from clinical samples and published genotypes showed the presence of distinct clusters. Overall, the majority of the samples analysed in this study were not closely related to any of the published sequences with the exception of samples TV357 and TV358 (Figure 14). The tree contained one cluster with only the clinical samples from this study with four samples from genotype G and the one sample from genotype I. This indicates that there was no significant diversity in the *actin* gene ORF of genotype I and certain genotype G samples. However, within the group of genotype G samples, a level of diversity was noted. Samples TV184, TV253 and TV357 did not cluster with the other genotype G samples. TV184 contained a single amino acid substitution (E→R) which was not present in the other genotype G samples. Sample TV253 did not contain any substitutions, however, it did not cluster with the other samples of genotype without substitutions. Sample TV357 was shown to cluster closely to the published G and H genotypes. This sample also carried two amino acid substitutions in the *actin* gene ORF (L→R) and (G →D). One of these substitutions (G →D) was also observed for one of the published genotype G strains (EU076578). Sample TV358 (genotype H) clustered on its own. This sample also contained a substitution (Q→K) in the *actin* gene ORF.

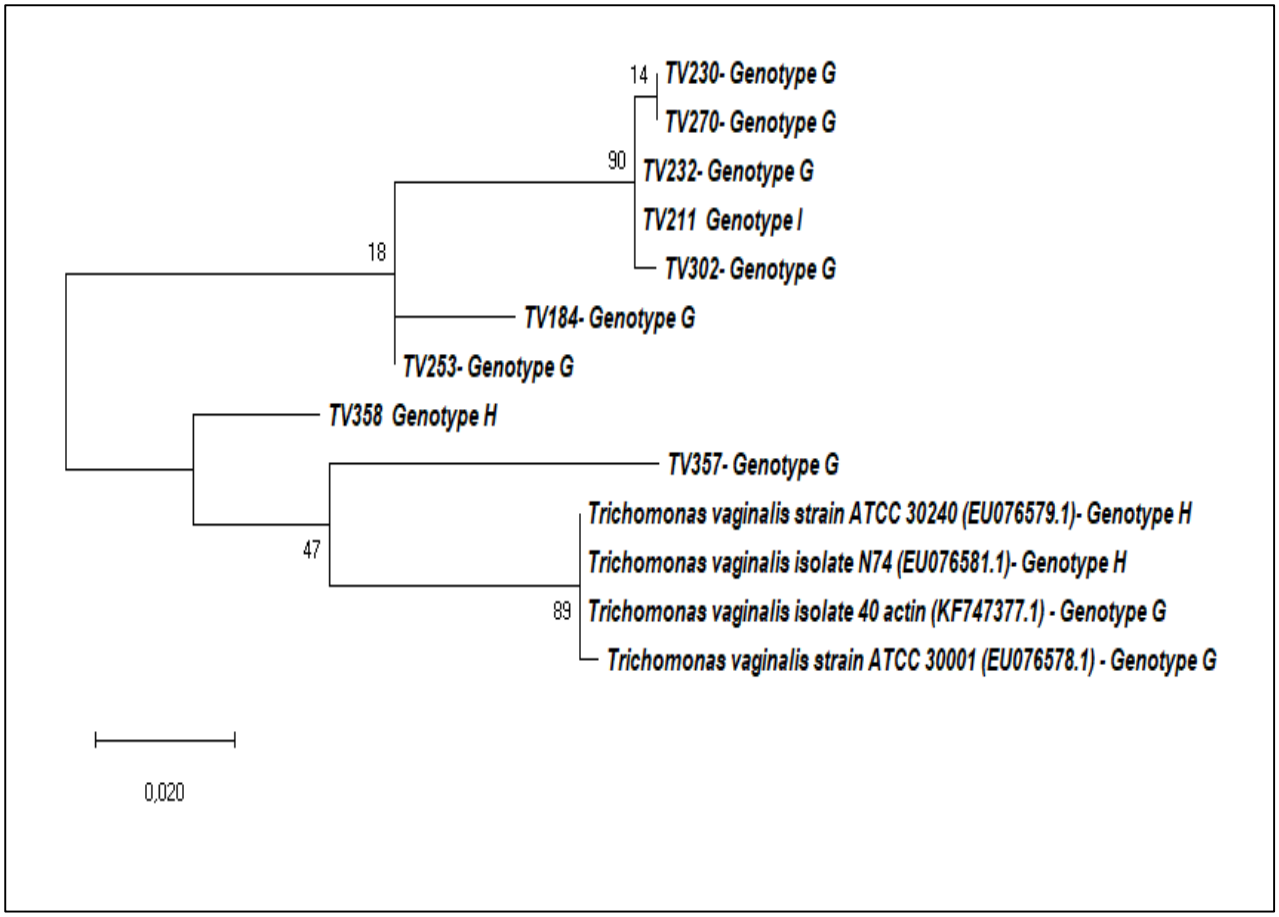

Figure 14: Phylogenetic tree of *Trichomonas vaginalis actin* genotypes according to the maximum-likelihood (ML), tree was conducted based on the multiple sequence alignment of *actin* gene by MEGA V10. Distance represents the number of base substitutions per site.

4.8 Distribution of genotypes in relation to clinical symptoms

For the one woman who carried genotype I, it was shown that this woman reported multiple symptoms associated with STIs such as abnormal vaginal discharge, foul-smelling vaginal odour, genital itching and genital warts (Table 7). In the studied population, there was also one woman who harboured genotype H. This woman was asymptomatic (Table 7). For the women who harboured genotype G, it was shown that a large proportion of these women (53.8%) were asymptomatic; followed by 23.0% who reported symptoms of abnormal vaginal discharge;

15.3% reported genital itching; and 7.6% reported a combination of symptoms (abnormal vaginal discharge and genital itching (Table 7).

Table 7: Genotypes linked to clinical symptoms of the studied population.

Genotype	Number of isolates	Clinical symptoms reported
I	1/1 (100%)	Abnormal vaginal discharged, foul- smelling vaginal odour, genital itching and genital warts
H	1/1 (100%)	No symptoms
G	7/13 (53.85%)	No symptoms
G	3/13 (23.08%)	Abnormal vaginal discharged
G	2/13 (15.38%)	Genital itching
G	1/13 (7.69%)	Abnormal vaginal discharged and genital itching

CHAPTER 5

DISCUSSION

Trichomoniasis is considered to be the most prevalent non-viral sexually transmitted infection (STI) worldwide affecting individuals of all ages, ethnicity and socioeconomic groups (Napierala *et al*., 2010; Conrad *et al*., 2012; Apalata *et al*., 2014; Naidoo, 2015). The global prevalence rates for *T.vaginalis* varies between 0.9%-80% (Valadkhani *et al*., 2008; Javanbakht *et al*., 2013; Abou-Kamar *et al*., 2017).

In this study, the prevalence of *T. vaginalis* in the antenatal women was approximately 13.0% (47/362). A similar prevalence rate of 10% for *T.vaginalis* was observed for antenatal women from Durban (Dessai *et al*., 2020). The prevalence estimates reported in South African women correlates with the rates reported for other African countries. A study conducted by Abou-Kamar *et.al*, 2017 in Egypt on pregnant and non-pregnant women, showed a prevalence rate of 13.5% for *T. vaginalis*. An earlier study conducted by the WHO in the African region in women aged 15 to 49 years of age reported an estimated prevalence rate of 11.5% for *T. vaginalis* infection (Newman *et al*., 2015).

Approximately 50% of women infected with *T. vaginalis* are asymptomatic (Sood and Kapil, 2008; Donbraye *et al*., 2010). In the present study, the majority of the antenatal women (57. 4%) were asymptomatic (no abnormal vaginal discharge) yet tested positive for *T. vaginalis*. Similarly, De Waaij *et al*., 2017, reported that 66% of the South African women in their study were asymptomatic.

Overall, the median age of the women who tested positive for *T. vaginalis* in this study was 28.0 (24.0-32.5). The majority of the study women had completed high school (68.1%), were unmarried (91.5%), had a regular sex partner (80.9%), had between 2-4 lifetime sex partners (66.0%) and were in the last trimester of pregnancy (59.6%). However, there were no

significant associations between these variables and *T. vaginalis* positivity. Shahraki *et al*., 2020 reported no relation between the *T. vaginalis* infection rates and educational level which is consistent with our findings. However, Munoz-Ramírez *et al*., 2018 reported that younger women (21-30 years) had the highest *T. vaginalis* infection rate when compared to women over 30 years of age. In a study by De Lemos and Garcia-Zapata, 2010, early sexual debut was associated with *T. vaginalis* infection rates and Donbraye *et al*., 2010, reported that the trimester of pregnancy may contribute to the prevalence rates of *T. vaginalis*.

The genetic characterization of *T. vaginalis* isolates from various regions show that there is significant genetic diversity in this organism (Meade and Carlton, 2013). Currently, there is a lack of data on the circulating genotypes of *T. vaginalis* in South African populations, particularly pregnant women. This was the first South African study, to employ the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of the *actin* gene to identify the different genotypes of *T. vaginalis* circulating in our population.

T. vaginalis is said to contain an actin protein which is coded by a 9-membered family, having nucleotide resemblances in 5 genes which are similar to mammalian *actin* genes. When *T. vaginalis* attaches to the surface of the host cell, it causes the parasite to transform from a tetanus form to amoebic form resulting in appendage formation i.e. pseudopodia (Shahraki *et al*., 2020). Both attachment and deformation regarding the host cell can be associated with the pathogenicity of *T. vaginalis*. The *actin* gene thus, plays a significant role in the parasite's pathogenicity thereby making it the gene of choice for molecular typing techniques (Rezaeian *et al*., 2009; Menendez *et al*., 2010; Shahraki *et al*., 2020).

In this study, three different genotypes of *T. vaginalis* were identified by PCR-RFLP of the *actin* gene. The most frequent genotype in our study population was the genotype G. Other *T. vaginalis* genetic studies conducted on the African continent revealed the presence of

genotypes G and E as highly prevalent (Crucitti *et al*., 2008; Masha *et al*., 2017). A study conducted in the Democratic Republic of Congo (DRC) and Zambia on female sex workers revealed the presence of eight different genotypes based on the *actin* gene. In the DRC, the most prevalent genotype was E, whereas in Zambia the most common genotype was G (Crucitti *et al*., 2008). Our study findings are consistent with the findings from Zambia. A study conducted on pregnant women from Kenya, reported a high frequency of genotype E (Masha *et al*., 2017) which is similar to the results obtained for the DRC (Crucitti *et al*., 2008). From the above-mentioned studies it is evident that common genotypes of *T. vaginalis* are circulating in various population groups across the African continent. In a study conducted in Ndola, Zambia involving adolescent girls, pregnant women as well as sex workers, Crucitti *et al*., 2010, identified nine different genotypes, with genotype G being the most frequent across all three study groups. This is similar to the findings of the pregnant women tested in the current study.

In studies conducted out of Africa, Momeni *et al*., 2015, identified five different genotypes with genotype G being the most prevalent in a population of Iranian men and women. However, three recent studies conducted in Iran (Matini *et al*., 2017; Khalili *et al*., 2017; Oliaee *et al*., 2017) observed a high prevalence of *T. vaginalis* genotypes which differed from the genotypes observed in Africa and those reported by Momeni *et al*., 2015 for Iran. Matini *et al*., 2017, investigated the prevalence of *T. vaginalis* genotypes in symptomatic and asymptomatic women, attending Gynaecology Clinics in western Iran. According to the *actin* gene digestion profiles, genotype A was shown to be the most prevalent in their study population (Matini *et al*., 2017). A study conducted on women seeking care in a general clinic in southwestern Iran, reported on genotype H as being the most frequent (Khalili *et al*., 2017). Similar findings were reported by Oliaee *et al*., 2017 in a population of incarcerated women from southern Iran where genotype H was shown to be the most frequent (Oliaee *et al*., 2017).

In this study, within the samples that belonged to genotype G, a level of diversity was noted as evidenced by the multiple sequence alignments and phylogenetic analyses. Three samples TV184, TV253 and TV357 did not cluster with the other genotype G samples. The possibility of obtaining different genotypes involving *T. vaginalis* within a study population could be due to large sample numbers as well as obtaining samples from regions which have a much higher prevalence rate (Abou-kamar *et al*., 2017). In this study, the prevalence of *T. vaginalis* was close to 13% and this relatively high prevalence could have contributed to the observed diversity. In addition, for the women who carried genotype G, it was shown that a large proportion of these women (53.8%) were asymptomatic; followed by 23.0% who reported symptoms of abnormal vaginal discharge; 15.3% reported genital itching; and 7.6% reported a combination of symptoms (abnormal vaginal discharge and genital itching). A study conducted by Khalili *et al*., 2017 also showed that in the group of women who carried genotype G there was a combination of women who were asymptomatic as well as symptomatic. It is possible that the genetic differences in the pathogen may contribute to the clinical manifestations associated with infection. However, this will need to be confirmed by further investigations.

Within the genotype G study samples, two of the three samples carried amino acid substitutions in the *actin* gene ORF. One particular sample TV357 showed a replacement of Leucine (L) with Arginine (R) and replacement of Glycine (G) with Aspartic acid (D). Similar findings were reported by Spotin *et al*., 2016 for a *T.vaginalis* isolate from Iran. The multiple alignment of the Iranian isolate which was assigned genotype G showed amino acid substitutions where Aspartic acid (D) replaced Glycine (G) and Arginine (R) replaced Leucine (L) (Spotin *et al*., 2016). This indicates that there may be a small level of similarity between *T. vaginalis* genotype G samples from different regions.

This study was limited in terms of the small sample size used for the genotyping analysis and sampling of only one region within KwaZulu-Natal. A larger sample size from a wider population is needed to obtain more substantial evidence on the diversity of this pathogen. This is a future study that our research group is embarking on. In addition, the *actin* genes were not shown to be present in all positive samples which is an unexpected finding, this could be due to technical errors during sample processing which will need to be investigated.

Future work emanating from this study, would be to investigate the association of the genotypes with patterns of drug susceptibility in this protozoa. In addition, it would be useful to compare the distribution of the genotypes in pregnant and non-pregnant populations. It may also be useful to look at the association of the genotypes with the prevalence of the different *T. vaginalis* viruses (TVVs).

CHAPTER 6
CONCLUSION

Early techniques which were applied for the typing of *T. vaginalis* isolates included, antigenic characterisation, monoclonal antibody binding (Krieger), karyotype polymorphism by pulsed field gel electrophoresis, random amplified polymorphic DNA (RAPD), and restriction fragment length polymorphism (RFLP). Despite the numerous list of typing techniques, each of the above-mentioned had significant limitations (Meade and Carlton, 2013). Currently, the PCR-RFLP technique based on the *actin* gene amplification provides a sensitive and reliable method for typing of *T. vaginalis* isolates (Oliaee *et al*., 2017).

The present study provides evidence on the genetic diversity of *T. vaginalis* from a South African pregnant population based on typing of the *actin* gene. Three different genotypes were observed in the studied population. Within the dominant genotype, i.e. genotype G, diversity in the *actin* gene ORF of the samples harbouring this genotype was observed. The observed diversity within specific populations can be challenging for future vaccine design and development of antigen-based rapid diagnostic tests for trichomoniasis. Future vaccine design studies and diagnostic studies will need to direct their focus on identifying antigenic determinants which are broadly representative of the entire *T. vaginalis* population (Meade and Carlton, 2013). However, one of our South African isolates was closely related to an Iranian isolate. This holds some promise that there may be conserved *T. vaginalis* isolates from different geographical regions. This could lend some hope for future vaccine design and diagnostic studies focused on identifying antigenic determinants which are broadly representative of the entire *T. vaginalis* population

CHAPTER 7

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CHAPTER 8

APPENDICES

Appendix I

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

04 November 2019

Miss Rennisha Chetty (214508419)
School of Clinical Medicine
Medical School

Dear Miss Rennisha Chetty,

Protocol reference number: BREC/00000406/2019
Project title: Genotyping of Trichomonas vaginalis in antenatal women from Durban
Degree: MMed

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given **full ethics approval** and may begin as from 04 November 2019. **Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.**

This approval is valid for one year from **04 November 2019.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **noted** by a full Committee at its next meeting taking place on **10 December 2019.**

Yours sincerely

Prof V Rambiritch (Chair)

Biomedical Research Ethics Committee
Prof V Rambiritch (Chair)
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Website: http://research.ukzn.ac.za/Research-Ethics/

Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

Appendix II

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

15 August 2018

Ms N Mabaso (211508336)
School of Clinical Medicine
College of Health Sciences
211508336@stu.ukzn.ac.za

Protocol: Next generation sequencing of metronidazole susceptible and resistant Trichomonas vaginalis clinical isolates from pregnant women from Durban, South Africa.
Degree: PhD **BREC Ref No: BE296/18**

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 02 May 2018.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 02 August 2018 to BREC letter dated 15 June 2018 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval** and may begin as from 15 August 2018. Please ensure that site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from **15 August 2018.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on **11 September 2018.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

cc postgraduate administrator: konarr@ukzn.ac.za
Supervisor: abbain@ukzn.ac.za Co-Supervisor: RamsuranV@ukzn.ac.za

Appendix III

Table 1: DNA Concentration (ng/µl) and DNA purity for positive *T. vaginalis* samples

Sample	TaqMan result	Actin result	DNA Concentration (ng/µl)	DNA purity
3	+	-	1.1	1.82
4	+	+	3.2	1.03
5	+	-	15.3	1.82
9	+	+	35.9	1.88
11	+	-	6.0	1.97
38	+	-	4.6	0.74
48	+	+	0.4	0.0
50	+	-	8.8	2.29
52	+	-	52.5	1.81
53	+	-	48.3	1.79
79	+	+	16.6	1.55
91	+	-	11.0	1.07
94	+	-	6.1	1.00
95	+	+	62.4	1.80
103	+	-	6.9	1.55
104	+	-	3.4	0.83
128	+	+	5.2	1.44
134	+	-	10.8	1.55
135	+	-	8.1	1.42
137	+	-	7.2	1.68
173	+	-	9.8	1.32
181	+	-	6.4	1.70
182	+	-	5.6	1.39
184	+	+	7.8	1.66
205	+	-	30.3	1.84
210	+	-	5.6	1.57
211	+	+	50.0	1.90
213	+	-	8.5	1.53
230	+	+	5.2	1.38
232	+	+	9.8	1.39
233	+	-	2.7	0.99
243	+	-	9.9	1.38
253	+	+	6.3	1.47
255	+	-	6.1	1.89
266	+	+	18.1	1.74
269	+	-	13.1	1.68
270	+	+	17.8	1.71
277	+	-	18.9	1.75
302	+	+	2.2	1.47
305	+	-	17.6	1.53
331	+	-	16.1	1.70
342	+	-	5.8	1.59
357	+	+	2.5	1.57
358	+	+	3.1	1.32

359	+	-	2.6	1.81
360	+	-	7.6	1.60
362	+	-	4.2	1.76

Symbols: (+) Positive

(-) Negative

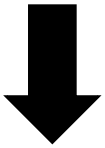
Actin positive results

Appendix IV

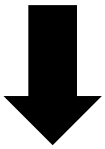
Flow Chart

T. vaginalis genotyping identification

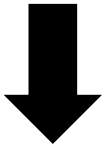
A total of 362 vaginal swab samples were obtained from pregnant women



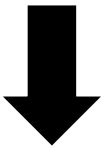
All 362 DNA samples were subjected to TaqMan quantitative PCR assay for the detection of *T. vaginalis*. A total of 47 of the 362 samples tested positive.



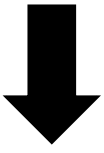
Positive samples 3, 4, 5, 9, 11, 38, 48, 50, 52, 53, 79, 91, 94, 95, 103, 104, 128, 134, 135, 137, 173, 181, 182, 184, 205, 210, 211, 213, 230, 232, 233, 243, 253, 255, 266, 269, 270, 277, 302, 305, 331, 342, 357, 358, 359, 360, 362



Positive samples were then used for the amplification of the outer and inner *actin* genes. Only 13/47 positive samples displayed bands at 1300 bp (outer gene) and 1100bp (inner gene), respectively.



The 13 samples were 4, 9,48,79, 95, 128, 184, 211, 230, 232, 253, 266, 270, 302, 357, 358



Genotyping of the *actin* gene was performed using the restriction fragment length polymorphisms (RFLP) technique. Restriction enzymes, *HindII*, *MseI*, and *RsaI* were used, resulting in the identification of three genotypes G, H and I.